Age-like Phenotype of Microglia During HIV-1 Infection

By

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Abstract

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HIV Associated Neurocognitive Disorder (HAND) persists in 50% of infected patients despite treatments. Moreover, HAND potentiates aging-associated neurocognitive decline as the infected population grow older. Microglia cells support productive HIV-1 infection in the brain and elevated senescence markers including p53 and p21 have been detected in microglia in HAND tissues. We hypothesize that HIV-1 induces age-like phenotypes in microglia. In an *in vitro* model of primary human microglia infected with HIV-1 pseudotype, we examined various markers of aging including the expression of cell cycle inhibitors (p53, p21 and p16), DNA damage associated p53 binding protein 1 (53BP1) foci formation, senescence associated β-galactosidase (SA-β-gal) activity, and the development of Senescence Associated Secretory Phenotype (SASP). Finally, we examined mitochondrial reactive oxygen species (ROS) and mitochondrial respiration, which are known to be altered during organismal aging. We detected elevated expression of p53 and p21, increased percentage of cells expressing 53BP1 foci and β-gal, as well as significantly elevated levels of various cytokines including IL8 and IL6 post infection. The development of age-

like phenotype is accompanied by increased mitochondrial ROS production and altered mitochondrial respiratory activity. Finally, exposure to the supernatant of infected microglia results in elevated p21 and caveolin-1 protein expression. Overall, we have demonstrated for the first time age-like phenotypes in human microglia during HIV-1 infection. HIV-1 induced microglial age-like phenotype could play a role in the development of HAND.

Chapter 1: Introduction

1.1 General Background

With the advent of combination anti-retroviral therapy (cART), which for most treated patients effectively controls HIV-1 viral replication, the proportion of patients developing HIV-1-associated dementia (HAD) has declined dramatically. However, recent population-based studies suggest that around 50% of all infected patients continue to develop HIV-1-associated neurocognitive disorders (HAND) to varying degrees (Chan and Brew 2014; Heaton et al. 2011; Nightingale et al. 2014; Sacktor et al. 2016). Remarkably, patients with even the mildest form of HAND -- asymptomatic neurocognitive impairments (ANI) -- have much higher chances of displaying impairment with daily activities due to cognitive decline as they age, compared to non-infected age-matched controls. This suggests that ANI potentiates age-associated cognitive impairment and having ANI is predictive of more serious impairment later on (Grant et al. 2014). Currently there are no effective treatments designed specifically for HAND and patients are managed with regimens commonly prescribed for patients with aging-related dementia such as antioxidants, N-methyl-D-aspartate (NMDA) antagonists and life style adjustments, such as exercising. More efforts are required to elucidate the molecular mechanisms underlying the development of HAND in order to design targeted therapies that could more effectively ameliorate the neurocognitive impairments.

Together with macrophages, microglia are the major cell type productively infected by HIV-1 in the CNS, and therefore it is also likely a major contributor to neurotoxicity observed during chronic HIV-1 infection (Gonzalez-Scarano and Martin-Garcia 2005). Various groups have proposed that the pro-inflammatory sequelae of microglia activation during HIV-1 infection, including altered cytokine secretion, comprise

a major tenant of HAND development (Gonzalez-Scarano and Martin-Garcia 2005; Lull and Block 2010). Advanced technology and dedicated efforts heralded significant advancement in our understanding of microglia physiology including unique molecular programs during microglial activation and aging, which could add new meanings to the implication of HIV-1 infected microglia during HAND.

The objective of the present review is to critically assess functional changes of microglia during chronic HIV-1 infection taking into consideration the aging demographics of the HIV-1-infected population, in order to identify molecular pathways that could serve as potential therapeutic targets.

1.2 Microglia Origin and Function

Introduced as the non-astroglial, non-neuronal "third element" of the CNS, microglia were first characterized in the 1930's by del Rio Hortega using silver staining technique (Rio-Hortega 1939). It was noted that microglial cells have the capacity to migrate, proliferate and phagocytose. Since then, advanced staining techniques and genetics-based studies elucidated that microglia belong to the myeloid phagocytic/monocytic lineage (Murabe and Sano 1982; Perry et al. 1985; Smith et al. 2013). Unlike other tissue resident macrophages, which are hematopoietically derived, the majority of microglia at steady state originate from a self-renewing cohort of yolk sac-derived erythro-myeloid progenitors (Ginhoux et al. 2013; Gomez Perdiguero et al. 2015; Sheng et al. 2015). Although there are significant variations in microglia density (0.5%-16.6%) and morphology depending on brain regions, whether region-dependent heterogeneity in microglia function exists remains largely unknown (Mittelbronn et al. 2001; Olah et al. 2011). Recent murine studies demonstrate differential genetic profiles depending on the brain regions, suggesting region-dependent functional differences (Grabert et al. 2016). At the basal level, microglia act as sentinels to survey the environment of the CNS through their motile processes and dynamic contacts with

neural cells, including astrocytes and neurons (Kettenmann et al. 2011). Recent evidence suggest that microglia prune pre-synaptic axons and post-synaptic dendritic spines to fine-tune synapses (Kettenmann et al. 2013; Tremblay et al. 2010). During neuronal development and maturation, a period of significant cell turnover, microglia also contribute to the clearance of dead cell debris through phagocytosis (Michell-Robinson et al. 2015). In the context of neurodegenerative diseases, microglial cells play essential roles in the clearance of beta amyloid (Aβ) proteins (Liu et al. 2010), the accumulation of which correlates significantly with neurocognitive impairments. Microglia express receptors for various neurotransmitters, cytokines as well as sensors for innate immunity including pattern recognition receptors such as Toll-like receptors (TLR) (Neumann and Wekerle 2013). Upon exposure to activating stimuli such as invading pathogens, microglial cells quickly mobilize to the site of insult and mount an innate immune response, and may help establish adaptive immunes responses, through phagocytosis, secretion of cytokines and chemokines, and antigen presentation (Aloisi 2001; Pascual et al. 2012; Shrikant and Benveniste 1996; Smith et al. 2010; Wraith and Nicholson 2012). Whether microglia activation is neurotoxic or neurotrophic is highly context-dependent. For instance, “acutely activated” (24 h treatment with the TLR4 ligand lipopolysaccharide [LPS]) microglia release pro-inflammatory cytokines and contribute to reduced neuronal survival rate, whereas “chronic activation” (72 h LPS exposure) of microglia induces secretion of anti-inflammatory cytokines and development of a neuroprotective phenotype (Cacci et al. 2008). As a result, the nature, duration and strength of microglial response to foreign insults are tightly regulated by inputs from both neural cells and components of the immune system. For example, depending on the type of stimuli, microglia could assume a pro-inflammatory/antigen-presenting activation state or anti-inflammatory/tissue-repairing activation state, similar to the M1 and M2 phenotypes traditionally used to describe macrophages, as demonstrated predominantly

with murine models (Ponomarev 2011) (Table 1). Evidence obtained with primary human cells suggests, however, that microglia initiate slightly different activation programs compared to that of monocyte-derived macrophages (MDMs). When stimulated with M1-polarizing agents such as interferon (IFN)γ, human adult and fetal microglia and MDMs all express elevated levels of CD80 and CCR7 with fetal microglia exhibiting the least elevation in CD80 and CCR7. On the other hand, treatment with M2 polarizing agents such as interleukin (IL)-4 leads to significantly increased CD23, CD163, CD206 and CD209 in MDMs, CD209 in adult microglia and CD209 and CD206 in fetal microglia (Durafourt et al. 2012). The different activation patterns between microglia and MDMs is just one of many functional characteristics that differentiate microglia from macrophages. For instance, adult microglia express qualitatively similar but quantitatively lower levels of antigenic markers including CD45, CD11b, HLA-DR, CD14, CD200R, CD16, CD32, CD64, CD86, CD163, CD68, CD4 and Ionized calcium-binding protein-1 (Iba-1) compared to macrophages (Kettenmann et al. 2011; Melief et al. 2012). Gene profiling and quantitative mass spectrometry analysis of murine cells demonstrate that microglia uniquely and highly express a group of transforming growth factor β (TGF-β)-dependent molecular signatures compared to other neuronal and immune cells, including resident macrophages in peripheral tissues (Butovsky et al. 2014). To conclude, microglia are an important and unique class of CNS-resident cells that perform various immune-modulatory functions to insure neuronal integrity. Disturbed microglial function has been attributed to the development of various neurological disorders including Alzheimer's, Huntington's, and Parkinson's, as well as HAND (Block et al. 2007; Nakajima et al. 2007).

1.3 Age-Dependent Changes in Microglial Function – Activation and Senescence

Microglial function evolves during the course of an individual's lifespan (Figure 1). Immuno-histochemical staining of brain sections of pre-term fetuses detected the presence of microglial cells in human CNS as early as 9 gestational weeks, which precedes birth and onset of hematopoiesis in the human bone marrow and liver (Verney et al. 2010). This observation is consistent with the discovery that the majority of adult microglia derive from embryonic progenitors, rather than from circulating monocytes generated during post-natal hematopoiesis. Fetal microglia strikingly differ from their mature, adult counterparts. In early gestational weeks, microglia appear amoeboid resembling activated microglia morphology and go through gradual ramification with increasing gestational age. Phenotypic analysis demonstrate that fetal microglia at pre-myelinating 16-20 weeks of gestational age express CD4, CCR5, CD11b, CD11c, CD14, CD45, CD68, CD86 and human leukocyte antigen-D-related (HLA-DR), and the staining intensity of antigenic markers weakens with increasing gestational age (Esiri et al. 1991; Rezaie and Male 1999; Wang et al. 2002). These observations suggest that fetal microglia have a partially activated state, which diminishes with maturation.

At the other end of the age spectrum, microglia also exhibit elevated activation status. Positron emission tomography (PET) demonstrated increased R-[^{11}C] PK11195 ligand binding, suggesting elevated microglial activation, in healthy aged humans (Schuitemaker et al. 2012). Additionally, molecular markers associated with microglia activation including major histocompatibility complex II (MHC II), CD11b and Iba1 have been shown to be elevated in microglia in tissues of aged individuals without overt neurocognitive deficits (Frank et al. 2006; Rogers et al. 1988; Ziv et al. 2006). The potential consequences of such chronically elevated activation of microglia during aging have garnered significant scientific interest in recent years, since the "microglial dysfunction hypothesis" suggests that age-related alteration of microglial function contributes to the onset of various neurodegenerative diseases (Wong 2013).

Another very important aspect of the age-associated alterations in microglia function is the development of the microglial senescence program. The concept of cellular senescence was first introduced by Hayflick and Moorhead to describe the limited lifespan of primary human cells in vitro, and has since been observed to play important roles in regulating human tissue aging and aging-related pathology (Hayflick and Moorhead 1961). It has been proposed that senescence is beneficial to reproductive fitness early in life through fine-tuning organ development during embryogenesis and as an anti-tumor mechanism by inducing permanent cell cycle arrest of neoplastic cells (Childs et al. 2014). As an organism ages, however, senescence appears to have detrimental health effects and contributes to the progressive loss of tissue and organ function during aging. More specifically, senescent phenotypes have been observed in various cell types residing in different tissues including bronchial cells, adipocytes and pancreatic β cells, as well as in astrocytes, and development of senescence in those cells has been shown to contribute to and/or to be associated with the onset of pulmonary fibrosis, obesity, type 2 diabetes and Alzheimer's disease, respectively (Bhat et al. 2012; Chesnokova et al. 2009; Chinta et al. 2013; Minagawa et al. 2011; Minamino et al. 2009; Sone and Kagawa 2005). Although it still remains to be clarified whether cellular senescence directly promotes aging, eliminating senescent cells appears to prolong the health span of both BubR1-hypomorphic progeroid and normal mice (Baker et al. 2016; Baker et al. 2011), and 'senolytic' drugs that can selectively kill senescent cells may potentially have remarkable beneficial effects in terms of health span and/or life span (Chang et al. 2016; Zhu et al. 2015a; Zhu et al. 2015b). It has been proposed that neutralization of detrimental effects of senescent cells or elimination of senescent cells could prevent the onset of various aging-related diseases, including neurodegenerative diseases (Salminen et al. 2011). As a result, much effort has been spent on characterizing senescence in various cell types in vivo and in vitro. Senescent

cells in vitro have many distinguishing characteristics including elevated expression of p53-p21 axis and/or $p16^{Ink4a}$ pathway that ultimately prevent entry into the S phase of the cell cycle; permanent DNA damage foci with accumulation of the p53-binding protein 53BP1 and/or the topologically-changed histone γH2Ax; increased enzymatic activity of the lysosomal hydrolase senescence-associated β-galactosidase (SA-β-Gal); and the development of the so-called senescence-associated secretory phenotype (SASP), which is a distinct secretory profile consisting of various pro-inflammatory cytokines, chemokines and metalloproteinases (Munoz-Espin and Serrano 2014) (Table 1). It was also shown that the onset of SASP is dependent on the initiation of DNA damage response and not present in all senescent cells (Rodier et al. 2009). The aforementioned traits need to be used in combination to characterize the senescent phenotype since any individual characteristic can be found independent of the initiation of the cellular senescence program. Cell culture experiments demonstrate that microglia undergo telomere shortening, which is a classic inducer as well as characteristic of senescence (Flanary and Streit 2004). Additionally, emerging evidence suggest that microglia could potentially develop a senescence-like phenotype with in vitro passaging. Compared to 2 days in vitro (DIV) microglia, 16 DIV murine microglia demonstrate elevated SA-β-Gal positivity, which might suggest the onset of cellular senescence (Caldeira et al. 2014). Importantly, transition to a potentially senescent phenotype correlated with reduced phagocytic and migratory capacities, indicating that what the authors describe as an "age-like phenotype" in microglia could result in impairment of its normal functions (Caldeira et al. 2014). However, it seems striking that the establishment of senescence may occur in microglia after a relatively short time of in vitro culture. In addition, the observation of reduced phagocytosis and mobility in these cells is not in agreement with very recent evidence obtained from aging mice using high spatial-resolution electron microscopy, which describes more active microglia in 14-month old than in 3-month old

mice. Imaging captured increased instances of microglia extending their processes to encircle neuronal synapses including pre-synaptic axon terminals and post-synaptic dendritic branches, which, together with the shrunken appearances of and empty spaces surrounding these neuronal processes, suggests active digestion of neuronal synapses by microglia in the aging mice (Bisht et al. 2016). These discrepancies could be due to the fact that microglia exhibit differential rates of phagocytosis depending on the objects being ingested – the in vitro study used beads for phagocytosis assay while the in vivo experiment observed microglial synaptic pruning, which is a more physiologically-relevant model. In addition, it is possible that sub-populations of microglia may exist in the aged brain, and that some may exhibit a phenotype more similar to that described in vitro. Although microglial senescence has not been examined systematically in human tissues, analysis of aged human brain tissues revealed the presence of dystrophic microglia, characterized as having altered cytoplasmic structures including de-ramified, tortuous processes with spheroidal bulbous swellings, which often progresses to fragmented cytoplasm (Streit et al. 2004). This dystrophic microglia phenotype is observed to increase with aging and has been detected in neuropathological conditions including Alzheimer's disease (AD) (Yang et al. 1998). Thus, the cumulative evidence suggests that aging is a significant modifier of microglial function. Although the progression and exact nature of microglial "aging" remains to be clarified, activation and senescence appear to be integral processes of the microglial "aging program".

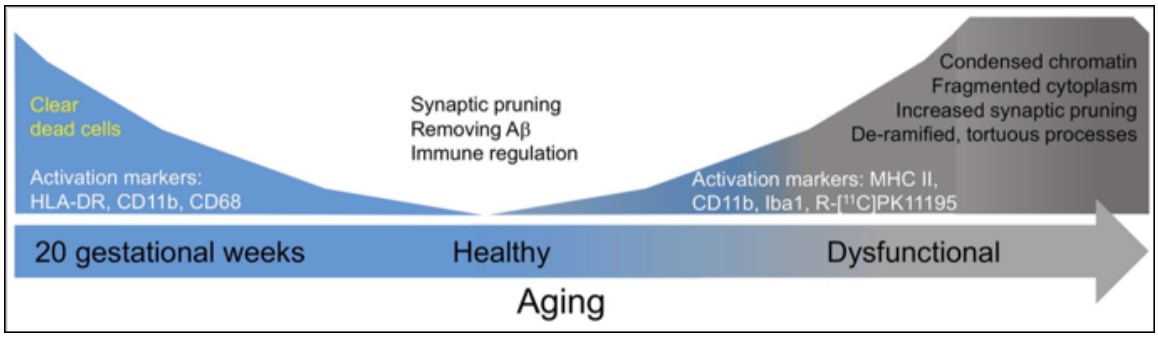

Figure 1. Age-dependent Changes in Microglia Microglia exhibit phenotypic changes over an individual's lifetime. During fetal development, microglia express activation markers including HLA-DR, CD11b and CD68, indicating an activation state (denoted by blue) that seems to be needed to accomplish proper differentiation and development, and clearing of dead cells. This activation state decreases with maturation and usually leads to a healthy, homeostatic status that predominates until later in life, when a build-up of chronic, persistent activation may occur. In this context, chronic activation may result in deleterious changes that associate with dysfunctional microglia (transition from blue to grey), characterized by morphological changes such as condensed chromatin and fragmented cytoplasm, and functional changes such as increased synaptic pruning that contributes to loss of neuronal synapses. This dysfunctional state may be reflective of a senescence phenotype and could contribute to the development of neurodegenerative diseases.

1.4 HIV-1 Infection and Viral Life Cycle

Since the first reported case of AIDS in 1981, much has been accomplished in characterizing the molecular and cellular mechanisms of HIV-1 infection, replication and pathogenesis in the past three decades (Barre-Sinoussi et al. 1983; Gottlieb et al. 1981). HIV-1 is an enveloped virus that contains two copies of single stranded RNA encased in capsid proteins. The HIV-1 viral genome encodes for nine structural and accessory proteins that play important roles in facilitating viral life cycle including env, gag, pol, tat, rev, nef, vpr, vif and vpu(Lu et al. 2011). In order to enter host cells, the envelope region of the virus first binds to the cell surface molecule CD4, which is the main receptor for HIV-1(Dalgleish et al. 1984; Maddon et al. 1986). Following binding with CD4, the envelope protein undergoes conformational changes, allowing subsequent interactions of the envelope with co-receptors including CXCR4 and CCR5, which are critical for the entry of X4 and R5 tropic viruses respectively(Feng et al. 1996). In fact, individuals deficient in co-receptor expression are protected from HIV-1 infection(Liu et al. 1996). Following receptor binding, the viral envelope or the lipid bilayer on the exterior of the viral particle fuses with the cell membrane and the viral capsid is delivered into the cytoplasm, where it uncoats to release the viral RNA and proteins. Positive sense viral RNA is reverse transcribed into DNA, which is then translocated into the nucleus in the pre-integration complex (PIC). In the nucleus, the viral DNA is integrated into the host genome and hijacks the host cellular machinery to transcribe and translate its own genome to form new viral RNA and viral proteins, which are then assembled into mature viruses to be released from the infected host cells. The released virion can then infect other cells(Nisole and Saib 2004). Our knowledge of HIV-1 viral replication has enable the discoveries and developments of successful antiviral treatments, which have transformed HIV-1 infection from a fatal disease in the early days of its discovery to now a manageable chronic disease. Most patients who are on effective treatments can have

a normal life expectancy(Barre-Sinoussi et al. 2013). Unfortunately, persistent immune-activation associated with viral infection and side effects of chronic treatments can result in various debilitating end organ diseases including neurological disorders. HIV associated neurocognitive impairments (HAND) can be diagnosed based on a battery of cognitive tests and categorized into asymptomatic neurocognitive impairment (ANI), mild neurocognitive disorder (MND) and HIV associated dementia (HAD) in increasing order of severity. Although the prevalence of HAD has significantly declined with effective treatments, around 50% of patients continue to develop the milder forms of HAND depending on the study. The neurocognitive complications of chronic HIV-1 infection remain a significantly public health problem in the post cART era(McArthur et al. 1993).

1.5 Persistent Viral Presence During cART Era

Despite stable courses of cART that may result in undetectable levels of virus in blood, HIV-1 persists in most infected patients and it is possible that the CNS may be one anatomical reservoir where the virus resides. Microglia constitute the only resident cells in the human brain parenchyma that can support productive HIV-1 infection and could thus serve as one of the potential cellular reservoirs within the CNS during chronic HIV-1 infection. This is supported by evidence demonstrating significant cerebrospinal fluid (CSF) viral escape in some patients on effective cART that have low or undetectable plasma HIV-1 RNA levels. In a retrospective analysis that examined viral load in patients' CSF and plasma, Eden et al. detected a median viral load of 121 HIV-1 RNA copies/ml in the CSF of 7 patients out of a total of 69 patients analyzed, all of whom had undetectable plasma viral load by standard assays (below 50 copies/ml) and were asymptomatic for neurocognitive diseases (Eden et al. 2010). In two additional retrospective studies that selected for patients who had developed neurocognitive deficits and brain magnetic resonance imaging (MRI) abnormalities, CSF viral escape (median viral loads of 880 and 3900 copies/ml, respectively) was detected in all patients

with undetectable plasma viral load (Canestri et al. 2010; Peluso et al. 2012). Furthermore, phylogenetic analyses have revealed compartmentalization of HIV-1 in the CSF compared to peripheral blood early post-infection, which suggests that HIV-1 evolves very quickly to adapt to the CNS microenvironment early on (Schnell et al. 2010; Sturdevant et al. 2012). This is in addition to the well-established compartmentalization of viral strains between brain and blood or lymphoid tissues determined with autopsy samples derived from patients with neurocognitive manifestations associated with HIV-1 infection (Gonzalez-Scarano and Martin-Garcia 2005). Furthermore, genetic and functional studies of the envelope gene of viral strains isolated from brain, and the comparison with those from blood or lymphoid tissues of the same infected patients, have demonstrated genetic changes that contribute to a reduced dependence on the primary receptor for infection, namely CD4; this reduced CD4 dependence confers these strains an increased ability to infect cells with low CD4 expression, compared to that of CD4+ T cells, such as microglia and macrophages (Gonzalez-Scarano and Martin-Garcia 2005; Martin-Garcia et al. 2006; Martin-Garcia et al. 2005; Rossi et al. 2008). The importance of macrophages and microglia during HIV-1 infection is further emphasized in a model of simian immunodeficiency virus (SIV)-infected rhesus macaques with depletion of CD4+ T cells, in which infection is sustained by macrophages and microglial cells (Micci et al. 2014). The above evidence suggests that HIV-1 has evolved to reside in long-lived cells such as microglia within the CNS, to achieve certain levels of viral replication refractory to immune pressure and antiretroviral therapies that, in most patients, effectively restrict viral load in the peripheral blood.

The presence of HIV-1 viral reservoir in the CNS is bolstered by the detection of stable SIV viral DNA in infected macaques even when viral RNA in circulation decreases to an undetectable level, suggesting a state of viral latency (Clements et al. 2011; Clements et al. 2005; Zink et al. 2010). Isolated microglia from SIV-infected animals and

HIV-1-infected human brains start to produce infectious virus upon in vitro culture, which further validate the observation that microglia harbor infectious HIV-1 virions (Brinkmann et al. 1993; Ghorpade et al. 2005). Overall, ex vivo and in vitro evidence suggest that the CNS, and microglial cells in particular, can potentially serve as one of the anatomical and cellular reservoirs, respectively, where HIV-1 may persist during chronic infection despite successful cART. As a result, it is important to better understand the interaction between HIV-1 and microglia during chronic HIV-1 infection.

1.6 The Effect of HIV-1 Infection on Microglial Function

1.6.1 Microglia activation during HIV-1 infection

Abundant clinical evidence suggests a state of immune activation in the CNS of patients infected with HIV-1(Table 1). Brain imaging of HIV-1-infected patients on cART using positron emission tomography imaging and 11C-PK11195 as an in vivo marker of microglia activation, reveals activation of microglia even in the absence of neurological symptoms (Garvey et al. 2013). In vivo and ex vivo studies of HIV+ patients demonstrate that increased immune activation in the CNS of infected patients compared to uninfected controls could be attributed to infected microglia (Andersson et al. 1998). Immunocytochemistry staining of brain sections from HIV-1-infected patients indicates that, compared to tissues from uninfected controls, there are significant increases in MHC class II, CD163, IL-1 and tumor necrosis factor (TNF)α levels, indicative of immune activation. The CSF from HIV+ patients also contains increased levels of TNFα, β2-microglobulin and neopterin, which are also markers of macrophage activation (Tyor et al. 1992). IL-1α was elevated in microglial cells derived from brains obtained from HIV-1-infected patients, and S100β, a cytokine that induces intraneuronal calcium levels, was also found to be elevated in the CNS of HIV-1-infected patients, which could contribute to neuronal degeneration (Stanley et al. 1994). Combined reverse

transcriptase/polymerase chain reaction, in situ hybridization and immunohistochemistry in brain and spinal cord sections of patients infected with HIV-1 demonstrate that cells of the macrophage lineage, including microglia, are positive for TNFα mRNA and therefore represent possible sources of TNFα in the brain during HIV-1 infection (Wesselingh et al. 1997). This was in contrast with the results of an ex vivo study utilizing microglia isolated from brain tissues of patients who were HIV seropositive. The cells were confirmed to be CD68+ and to express p24 antigen by immunofluorescence and were subsequently stimulated with LPS for 24 h. Surprisingly, HIV-1-infected microglia secreted lower levels of TNFα both in basal conditions and post-LPS challenge than control microglia, suggesting that microglia chronically infected by HIV-1 may exhibit subdued pro-inflammatory profile compared to uninfected cells, despite the fact that most of the microglia stain positive for HLA-DR, indicative of activation (Ghorpade et al. 2005). Unfortunately, a more extensive secretory profile was not investigated to fully examine the activation status of infected microglia in this particular study.

Immunohistochemistry staining of brain sections from chronically-infected patients who have developed HIV-1 encephalitis (HIVE) revealed elevated levels of IL-1 and caspase-1 in microglia, which suggests that inflammasome activation, as a part of the microglia activation program, could contribute to the development of HAND (Zhao et al. 2001). This was confirmed in an in vitro study in which HIV-1 infection resulted in inflammasome activation, as indicated by the release of IL-1β and caspase-1 activation (Walsh et al. 2014). Using an in vitro primary human microglia model, HIV-1 infection results in secretion of IL-8, IL-6, monocyte chemotactic protein 1 (MCP-1), TNFα and RANTES (or “regulated on activation, normal T cell expressed and secreted”), in a Beclin-1-dependent manner (El-Hage et al. 2015). Microglial activation is also observed in animal models of HIV-1 infection of the CNS. In brain tissues of rhesus macaque

monkeys infected with SIV, in situ hybridization showed that a majority of infected microglia expressed MHC class II, indicating glial activation (Brinkmann et al. 1993). Astroglial activation, as indicated by microglial nodule formation and increased IL-1β and IL-6 expression, was also observed in severe combined immunodeficiency (SCID) mice that developed encephalitis upon receiving xenografts of HIV-1-infected cells (Persidsky et al. 1996). Although most of the evidence suggest that microglial cells predominantly exhibit pro-inflammatory status in the context of HIV-1 infection, surface marker analysis on brain sections from patients with HIVE detected elevated levels of CD163 in association with ramified microglia, suggesting a de-activated or alternatively-activated phenotype (Roberts et al. 2004).

All evidence considered, it is likely that microglia exist in a spectrum of activation status in the context HIV-1 infection, however the mechanisms regulating differential microglial activation during the course of HIV-1 infection remain largely unknown. Pro-inflammatory profiles seen in microglia during HIV-1 infection could be due to multiple factors including infection itself, viral protein-mediated or caused by cytokine secretion, possibilities which can be further explored in an in vivo setting. Using a primary human fetal microglia model, it was shown that HIV-1 integrase inhibitor Raltegravir significantly reduced the levels of multiple cytokines including IL-8, IL-10, TNFα and IL-6, which suggest that the infection definitely plays an important role in either the initiation or propagation of the pro-inflammatory phenotype (Tatro et al. 2014).

1.6.2 HIV-1 infection affects the apoptosis vs. survival pathway of microglia

HIV -1 infection also affects the balance of microglial survival and death. Recent evidence suggests that terminally differentiated tissue macrophages have the potential to proliferate in order to self-maintain under favorable conditions (Hashimoto et al. 2013)(table1). The intricate balance of this process is perturbed during HIV-1 infection,

since it was shown to inhibit granulocyte-macrophage colony-stimulating factor (GM-CSF)-mediated CD68+ macrophage/microglia proliferation observed in vitro as well as in brain tissues using Ki67 and BrdU (5-bromo-2'-deoxyuridine) staining (Cosenza-Nashat et al. 2007). In vivo and in vitro evidence also suggest that productive HIV-1 infection promotes a pro-apoptotic environment by up-regulating BCL2-associated X protein (Bax) (pro-apoptotic) and inactivating B-cell lymphoma (Bcl)-2 and Bcl-x (anti-apoptotic) (Krajewski et al. 1997). However, it is unclear whether microglial apoptosis occurs, and how widespread it is, during HIV-1 infection. On the other hand, there have been also studies demonstrating that microglial cells avoid HIV-1 infection-mediated cell death through up-regulation of pro-survival pathways. For instance, primary human microglia promote cell survival by up-regulating BCL2 Associated Athanogene (BAG) 3, whose down-modulation seems to be required for caspase-3-mediated apoptosis following infection (Rosati et al. 2009). Other studies suggest that HIV-1 infection could modulate cell survival at the level of cell cycle regulation. For example, an aberrant profile of cell cycle regulators was observed using the SIV model. In brain tissues with SIV encephalitis, levels of E2F1 and retinoblastoma susceptibility gene product (pRB) were shown to be elevated in neurons and glial cells in both frontal cortex and basal ganglia using immunofluorescence staining (Jordan-Sciutto et al. 2000). E2F and pRB are required for the initiation of S phase during cell cycle progression; however, in terminally differentiated cells such as neurons, E2F could cooperate with p53 to initiate other p53-mediated cellular processes including apoptosis (Pan et al. 1998). Unfortunately, expression of proteins mediating apoptosis such as caspase-8 and -9 was not examined to determine the potential link between E2F1 up-regulation and apoptosis during infection. In subsequent studies, no difference was detected in E2F target genes including proliferating cell nuclear antigen (PCNA) and cyclin A, which are important for cell cycle progression, or $p19^{ARF}$, which mediates p53-dependent apoptosis in neurons

(Wang et al. 2010). Thus, the consequences of altered cell cycle proteins including E2F and pRB in neurons during HIV-1 infection remain to be fully clarified.

Additionally, it has been shown that the level of MDM4, a homologue of E3 ligase murine double minute 2 (MDM2) that negatively regulates p53 activity, is reduced in the brain tissues of HIV-1-infected patients and patients with HAD (Colacurcio et al. 2013). This was consistent with up-regulated protein levels of p53 in both HIV-1-infected human and SIV-infected macaque brain tissues (Garden et al. 2004; Jayadev et al. 2007; Jordan-Sciutto et al. 2002; Jordan-Sciutto et al. 2000). In addition to an elevated p53 signaling pathway, target genes of p53, including p21 and Bax, were both up-regulated in neurons and glial cells in HAD tissues (Jayadev et al. 2007). Although p53 was up-regulated in both astrocytes and microglia identified with cell type-specific markers, no distinction between the two glial cell types was made regarding the expression of its target genes. To conclude, the levels of various cell cycle regulators are modulated in HIV-1-infected tissues and appear to be associated with the onset of HAND. Specifically, the p53-p21 pathway, which typically inhibits cell cycle progression, is elevated in various CNS cell types, including microglia, during infection. Although the p53-p21 axis is well known for its role in cell cycle regulation, evidence suggests its relevance in other cellular pathways in a cell type-dependent manner. In addition to regulating the expression of cell cycle inhibitors, p53 was also shown to control macrophage activation (Su et al. 2013). In murine models, p53 knock-out abrogates IFNγ-induced microglia activation, which suggests that the p53 pathway also promotes activation of microglia (Jayadev et al. 2011). Therefore, it is important to consider the effect of elevated p53 on microglia activation, in addition to cell cycle progression, during HIV-1 infection. Although, the consequential impact of the altered cell cycle machinery remains to be clarified, HIV-1 infection or bystander effects of HIV-1 infection seem to disrupt the delicate balance of

cell survival, cell cycle progression and apoptosis, which could contribute to the development of HAND.

1.6.3 HIV-1 infection affects cellular metabolism in microglia

Individuals with chronic HIV-1 infection often display severe metabolic disorders including disturbance in protein turnover, insulin resistance and altered amino acid metabolism (Gostner et al. 2015; Hommes et al. 1990; Vigouroux et al. 1999). Metabolomic screening of CSF from HIV-1-infected patients detected elevated levels of metabolites typically associated with mitochondrial dysfunction (succinate) and microglial activation (glutamate and arachidonate) in patients with HAND compared to those without any neurocognitive impairment (Cassol et al. 2014). Elevated kynurenine pathway downstream of tryptophan metabolism has also been shown to be associated with neurocognitive impairments during HIV-1 infection (Kandanearatchi and Brew 2012). Increased concentrations of kynurenine pathway metabolites such as quinolinic acid and heightened activities of the kynurenine-catabolizing enzymes have been detected in brain tissues as well as CSF of HIV-1-infected patients and SIV-infected macaques (Heyes et al. 1998; Sardar et al. 1995; Valle et al. 2004). Microglia is a major source of CNS quinolinic acid, which can induce neurotoxicity as an NMDA receptor agonist. Importantly, increased production of quinolinic acid is correlated with microglial activation and contributes to neurotoxicity during HAND (Drewes et al. 2015; Guillemin et al. 2005; Heyes et al. 2001). Infection of primary human microglia using neurotropic HIV-1 also results in the release of quinolinic acid and, in return, quinolinic acid treatment seems to promote HIV-1 viral replication and elevated CCR5 expression in microglia (Chao et al. 2000; Kandanearatchi and Brew 2012). Thus, altered kynurenine metabolism in microglia during HIV-1 infection could contribute to HAND development.

In addition to increased amino acid metabolism, activation of autophagy, which is a catabolic process that recycles cellular components in the event of nutrient starvation,

is also observed in microglia during HIV-1 infection. In primary human microglia cells, HIV-1 infection induces activation of autophagy in a time-dependent manner, and knock-down of beclin 1, which is required for multiple stages during autophagy, results in reduced p24 release as well as reduced secretion of pro-inflammatory cytokines (El-Hage et al. 2015). This study suggests that autophagy activation is essential for viral replication as well as infection-mediated immune activation of microglia cells (El-Hage et al. 2015). In a large-scale small interfering RNA screen, various proteins essential for regulating autophagy, including autophagy-related factors (Atg)7, Atg8 and Atg16L2, had been found to be required for HIV-1 infection (Brass et al. 2008). This had been also confirmed in T cell lines with stable knock-down of various Atgs (Eekels et al. 2012). The dependency of HIV-1 replication on autophagy was highly unexpected since autophagy has always been considered as an effective immune defense mechanism against intracellular pathogens, including viruses, by promoting the degradation of viral materials (Talloczy et al. 2006). It is important to note, however, that HIV-1 infection activates the initiation but inhibits the maturation of autophagy, to simultaneously promote autophagy-dependent processing of viral materials and inhibit degradation of viral proteins by autophagy in macrophage lineage cells (Kyei et al. 2009). Interrupted autophagic processes could compromise the turnover of various cellular organelles including the mitochondrion. As a result, abnormal autophagic activation is detrimental to mitochondrial health, which is also observed during HIV-1 infection (Lee et al. 2012; Miro et al. 2004; Miro et al. 2003). In fact, abnormal mitochondrial fusion and fission, which are critical processes for maintaining overall mitochondrial dynamics, have been observed in the brains of patients with HAD compared to HIV-1-infected but neurocognitively normal subjects (Fields et al. 2016b). Specifically, the protein levels of dynamin 1-like protein, which is a GTPase that promotes mitochondrial fission, are reduced in the frontal cortex tissue of patients with HAD (Fields et al. 2016b).

Accelerated mitochondria turnover by activating autophagy was sufficient to reverse microglial activation in a preclinical, transgenic murine model of HAND (Fields et al. 2013; Fields et al. 2016a). Taken together, these results suggest a role for altered mitochondrial homeostasis in microglial dysfunction during HAND.

Interestingly, the altered metabolomic profile, inefficient autophagy activation and reduced mitochondrial turnover observed in the CNS during HIV-1 infection are comparable to what may occur during the aging process, which suggests that altered cellular metabolism in microglia may underlie accelerated aging in HIV-1 infected patients with neurocognitive impairment (Cassol et al. 2014; Torok et al. 2016)(Table 1).

1.6.4 Microglia processing of Aβ protein during HAND

Clearance of Aβ protein constitutes an essential function of microglia cells. Amyloid protein can undergo either the non-amyloidogenic or amyloidogenic pathways. In the amyloidogenic pathway, amyloid precursor protein (APP) is enzymatically processed to produce $A\beta_{42}$ or $A\beta_{40}$, which can then form oligomers and insoluble fibrils. Elevated Aβ fibril deposition in brain tissues and correlatively reduced CSF soluble $A\beta_{42}$ levels have been described as hallmarks of some neurodegenerative diseases including AD (Aguzzi and O'Connor 2010). Intracellular Aβ accumulation has also been linked to neuronal damage during aging and development of AD (Baker-Nigh et al. 2015). However, clinical studies on amyloid homeostasis during HAND development are not as clear-cut. While some groups have described reduced CSF level of $A\beta_{42}$ comparable to that of patients with AD (Brew et al. 2005; Krut et al. 2013), others have found no significant differences (Ances et al. 2010; Ances and Ellis 2007). Studies that report significantly reduced CSF $A\beta_{42}$ levels examined subjects with HAD, whereas those that do not report reduction enrolled patients with HAND encompassing all stages, including the mildest form. It is possible that CSF $A\beta_{42}$ reduction is only detectable in the latest

stages of HAND. All studies on CSF $A\beta_{42}$ examined patients with mean ages of less than 50 yrs old, and it is unclear whether an older demographic would yield different results. Furthermore, MRI studies do not detect elevated $A\beta_{42}$ fibrils in patients with HAND compared to cognitively normal HIV+ or HIV- controls, and no difference was detected between cognitively normal HIV+ and HIV- subjects across all age groups (between 35 and 65 years of age), which suggest that $A\beta_{42}$ fibril formation is not altered due to HIV-1 infection and is not associated with HAND development (Ances et al. 2012; Ortega and Ances 2014). Our understanding of amyloid metabolism during HIV-1 infection is further clouded by conflicting reports on the association between HAND development and Apolipoprotein E (ApoE) expression, which is a significant risk factor for abnormal amyloid deposition resulting in sporadic AD development (Kim et al. 2009; Morris et al. 2010). While some early reports demonstrate increased frequencies of HAD development in ApoE carriers, more recent studies do not find a correlation between ApoE phenotype and HAND development characterized by either neurocognitive impairments or detrimental neuroimaging outcomes (Burt et al. 2008; Cooley et al. 2016; Corder et al. 1998; Morgan et al. 2013; Valcour et al. 2004b). These differential results could be due to disparities in stages of neurocognitive impairments examined (HAD vs. milder forms of HAND) or in ages of study subjects since ApoE was shown to be an independent risk factor for HAD in HIV-infected patients who are 50 year of age and older but not in younger subjects (Valcour et al. 2004a). ApoE status does not modify amyloid deposition during HAND in a cohort largely composed of patients younger than 50 years old (Ances et al. 2012). The distribution of amyloid protein in the CNS of HAND patients has also been examined through immunofluorescence staining of human brain tissues. Using antibodies targeting APP/$A\beta$, both intraneuronal and extracellular diffuse APP/$A\beta$ accumulation has been detected in the brains of HIV-1-infected patients (Green

et al. 2005; Nebuloni et al. 2001). HAND patients do not display the typical plaques of Aβ fibrils observed in AD patients. However, the increased intracellular as well as extracellular diffuse Aβ accumulation detected in HAND patients suggests an increased pool of soluble Aβs, which could be associated with the presence of dysfunctional microglia.

1.7 The Effect of Viral Proteins on Microglial Function

In addition to their susceptibility to infection by HIV-1, microglia in vivo are also exposed to several viral proteins such as Tat, gp120, Vpr and Nef, which are released by infected cells and can be detected in the CNS (Chang et al. 2011; Mukerjee et al. 2011). Various studies suggest that exposure to those viral proteins may also result in altered microglial function and that these effects could underlie the microglial dysfunction observed both in vivo and in in vitro models of infection.

Tat is the trans-activating protein of HIV-1 that plays a vital role in HIV-1 viral transcription and has been shown to affect multiple cellular functions, thereby contributing to the pathogenesis of HIV-1 infection (Romani et al. 2010). An in vitro study exposing microglial cells to increasing concentrations of Tat demonstrated a dose-dependent stimulation of the secretion of cytokines and chemokines (Sheng et al. 2000). When exposing microglia to Tat in the presence of specific inhibitors of various signaling pathways, it was demonstrated that Tat stimulates the secretion of MCP-1 and macrophage inflammatory protein (MIP)-1β by activating extracellular-signal-regulated kinases (ERK)1/2 MAPK; IFNγ-induced protein (IP)-10 through phosphatidylinositide 3-kinase (PI3K) activation; and IL-8 through p38 MAPK activation (D'Aversa et al. 2004). Tat also stimulates the production of MCP-1, IL-1β, TNFα and inducible nitric oxide synthase (iNOS) in a cyclooxygenase (Cox)-2-dependent manner, downstream of NF-κB activation (Flora et al. 2006). Finally, activation of nicotinamide adenine dinucleotide

phosphate (NADPH) oxidases also promotes the secretion of various pro-inflammatory cytokines and associated neurotoxicity following Tat exposure (Bokhari et al. 2009; Jadhav et al. 2014; Turchan-Cholewo et al. 2009).

In addition to pro-inflammatory cytokines, Tat activation of p38, p42/44 MAPK and NADPH oxidase pathways leads to increased glutamate release, which could potentially induce excitotoxicity and further contribute to neurotoxicity (Gupta et al. 2010). Tat exposure also promotes microglial migration towards, and phagocytosis of, axonal arbor, resulting in loss of neuronal synapses (Eugenin et al. 2005; Marker et al. 2012).

The surface gp120 sub-unit of the viral envelope glycoprotein is non-covalently bound to the transmembrane sub-unit gp41, and is known to shed in CD4-dependent and –independent manners from viral envelope glycoprotein trimers present both in the surface of virions and in the plasma membrane of infected cells (Ruprecht et al. 2011; Selhorst et al. 2013). This soluble gp120 can engage receptors in multiple cells and could thus result in activation of microglia through diverse mechanisms. Exposure to gp120 results in up-regulation of intercellular adhesion molecule (ICAM)-1 expression in a human astroglioma cell line and primary rat microglia, mediated by protein kinase C and tyrosine kinase (Shrikant et al. 1996). Exposure of microglia to gp120 also induces an outward K+ current and a concomitant increase in voltage-gated K(v) channel proteins, which could lead to elevated cytokine secretion, and subsequently contribute to neuronal damage that can be attenuated with K(v) channel blockers (Xu et al. 2011). In rat microglia, it was further shown that gp120 enhancement of voltage-gated K(v) channels depends on the activation of p38 MAPK pathway (Liu et al. 2012). Exposure of microglial cells to gp120 has also been recently shown to induce inflammasome activation and associated IL-1β secretion (Walsh et al. 2014).

Although these in vitro studies reveal important information on the effects of viral proteins on cellular functions, and specifically in microglia, their physiological relevance in vivo needs to be critically assessed, especially with regard to the concentrations of viral proteins used in vitro. In in vitro studies, concentrations of Tat in the range of 100 to 1000 ng/ml are routinely used; this is much higher than the concentrations of Tat detected in sera of HIV-1-infected patients, which usually ranges from 1 to 40 ng/ml depending on the study (Westendorp et al. 1995; Xiao et al. 2000). It is also possible that Tat release from infected cells may lead to locally higher concentrations, but these have not been demonstrated to date. Similar criticisms can be made for the studies utilizing gp120 proteins. While earlier studies may have detected higher serum concentrations of the envelope glycoprotein gp120 (Gilbert et al. 1991; Oh et al. 1992), more recent studies reported lower levels in the plasma of HIV-1-infected patients in the range of 0.5-15.6 ng/ml (Rychert et al. 2010), and even undetectable amounts in serum samples from patients with low-to-undetectable viremia (Santosuosso et al. 2009). It has also been shown in high viral load, rhesus macaques acutely-infected with a simian-HIV (or SHIV, encoding the HIV-1 envelope glycoproteins in an SIV genome) that HIV-1 gp120 was present in a much higher concentration in lymph node lysates than in plasma (218 ng/ml vs. 1.5 ng/ml, respectively) (Stevceva et al. 2008). However, in another study, cell-free tissue extracts from lymph nodes and spleens from patients with low-to-undetectable viremia showed that gp120 was undetectable or below 9 ng/ml (Santosuosso et al. 2009). To our knowledge, no study has quantified gp120 levels in brains of HIV-1-infected patients, but because one would expect the number and proportion of infected cells in the brain to be lower than in lymph nodes or spleens, it seems likely that a lower concentration of gp120 would be found in the brain than in those other tissues. However, concentrations of 200-500 ng/ml of gp120 are regularly used in in vitro studies, and these seem to be well above what might be expected in the

brain. In addition, the vast majority of in vitro studies have used the soluble gp120 of a CXCR4-using strain, HIV-1_{IIIB}, while the viruses isolated from brain tissues are almost exclusively using the alternative co-receptor for infection, namely CCR5. This is because infection of macrophages and microglia is only efficient for a subset of CCR5-using viruses, and for a few exceptions among the CXCR4-using strains. Thus, rather than focusing on the effects of IIIB gp120 in microglia, more studies should be directed to address the effects of macrophage/microglia-tropic, CCR5-using gp120 proteins from brain- or CSF-derived isolates.

Any effects of viral proteins on HIV-1 disease progression in patients likely occur through chronic exposure at physiologically relevant concentrations, which are conditions that cannot be appropriately modeled with in vitro cell culture systems that most often use higher concentrations over acute time courses. To complement results obtained using in vitro models, in vivo animal models treated with or expressing viral proteins for longer periods of time at lower concentrations have been used to study the effects of viral protein exposure during chronic HIV-1 infection. In a non-infectious HIV-1 transgenic rat model that expresses 7 of 9 HIV-1 viral proteins including gp120, Tat and Nef, microglia activation characterized by elevated Iba-1 expression is detected in younger groups and declines with age (Reid et al. 2016). Elevated Iba-1 expression post-gp120 exposure is also demonstrated using a well-characterized transgenic mice model expressing soluble CXCR4-using HIV-1 LAV gp120, and could be reversed by modulating pathways that facilitate mitochondria regeneration (Fields et al. 2013; Fields et al. 2016a; Maung et al. 2014; Toggas et al. 1994). In a rat model that has been transduced to express HIV-1 NL4-3 gp120, also a CXCR4-utilizing envelope, microglia activation peaks at week two and then transitions into the onset of apoptosis as indicated by significant TUNEL staining in CD68+ cells at one and three months, which can be partially protected with antioxidant treatment (Louboutin et al. 2009; Louboutin et

al. 2010), suggesting that activated microglia might eventually undergo apoptosis during protracted gp120 exposure, possibly due to prolonged oxidative stress. Results generated using these models have limited impact, however, considering that, as discussed above, gp120s from CCR5-utilizing viral strains are more relevant for studying effects on microglia function during HIV-1 infection, and the concentrations of gp120 obtained in both models have not been examined.

Microglia activation is also observed using various in vivo models of Tat exposure. For example, microglia activation was detected in mice that were injected with Tat in the striatum for 7 days (El-Hage et al. 2006; Puccini et al. 2015), and in the brain tissues of a rat model two weeks post-stereotaxical Tat injection in the striatum as well (Agrawal et al. 2012). Microglia activation upon Tat exposure has been reversed with over-expression of antioxidant enzymes including Cu/Zn superoxide dismutase (SOD1) and glutathione peroxidase (GPx1), suggesting a role of oxidative stress in Tat-induced microglia activation (Louboutin et al. 2014; Louboutin and Strayer 2014). An important caveat of the Tat injection models in mice and rats is that the initial injected concentrations of Tat are usually much higher than the physiological levels, although the actual final concentrations of Tat in the animal brains could be significantly lower due to distribution to wider areas as well as Tat degradation in cells and tissues over time (Passiatore et al. 2009). Another limitation of the stereotaxical injection models is the localized effects around the injection site, which has been the striatum in all studies. While the striatum is a brain area with high rate of productive HIV-1 viral replication, other areas such as the frontal gyrus are also affected during HAND development and should be examined during Tat exposure (Glass et al. 1995). Compared to direct stereotaxical injection of Tat in animal brains, a more physiologically-relevant model appears to be the conditional Tat transgenic mouse model that could be induced to express Tat in astroglial cells at 0.01-0.85 ng/ml. Three months of Tat expression in this

model also results in elevated microglia activation evidenced by nitrosative cellular stress (Hahn et al. 2015; Kim et al. 2003).

In addition to gp120 and Tat, other viral proteins including Nef and Vpr have also been shown to stimulate production of pro-inflammatory cytokines in microglia as a result of oxidative stress (Si et al. 2002; Vilhardt et al. 2002). Overall, HIV-1 viral proteins affect multiple functions of microglia including activation, migration, phagocytosis and glutamate secretion, all of which are relevant aspects of microglia dysfunction during HIV-1 infection and could contribute to the development of HAND. While directly exposing microglia in vitro to viral proteins does not necessarily recapitulate all biological processes during HIV-1 infection, this approach does afford us the opportunity to dissect mechanisms underlying functional changes of both infected and uninfected microglia. This type of studies has revealed that many of the phenotypes observed during HIV-1 infection could be due to activation of various cellular processes that mitigate oxidative stress responses, including inflammasome, p38, p42/44 MAPK, NADPH oxidase and NF-κB signaling, many of which are known to facilitate cellular senescence (Freund et al. 2010) and could be the pathways upon which HIV-1 infection and activation of cellular senescence might converge.

Table 1. Altered Microglial Functions During HIV-1 Infection

	HIV-1 infection/Viral proteins	M1 polarized	M2 polarized	Aging	Senescence
Immunological profile	Pro-inflammatory cytokines Chemokines Metalloproteinases Reduced phagocytosis R-[^{11}C]PK11195	IL-6, IL-8 and TNFα	IL-10 and TGF-β	Immune amplification Reduced endocytosis R-[^{11}C]PK11195	Pro-inflammatory cytokines Chemokines Metalloproteinases
Cellular metabolism	Induction of autophagy Elevated glutamate	Elevated glycolysis	Elevated mitochondrial respiration (β-oxidation)	Elevated lipid synthesis	Altered autophagy Impaired mitochondrial function
Cell cycle regulation	Elevated p53, p21, and E2F Reduced MDM4				Elevated p53, p21, and p16

1.8 Concluding Remarks

With the aging of the HIV-1-infected population in the cART era, health care professionals working with HIV-1-infected patients are faced with new challenges of treating both aging-associated loss of functions and health risks, as well as the chronic viral infection and inflammation associated with it. In addition, it seems well established now that the latter seem to have an effect on the former. Although a large recent study suggested that HIV-1 infection may cause accentuated but not accelerated aging (Althoff et al. 2015), many others have presented evidence of accelerated aging in chronically HIV-1-infected populations (Angelovich et al. 2015; Cassol et al. 2014; Chou et al. 2013; Gianesin et al. 2016; Horvath and Levine 2015; Levine et al. 2015; Martin et al. 2013; Pathai et al. 2014; Pathai et al. 2013a; Pfefferbaum et al. 2014; Schrack et al. 2015), involving multiple immune system- and CNS-related functional deficits. This review highlighted the many commonalities between functional changes of microglia during HIV-1 infection and during aging, including cellular activation, arrested cell cycle progression and altered cellular metabolism. Although microglia exhibit various signs of activation including secretion of elevated levels of pro-inflammatory cytokines, we do not think that activation captures the complexity of altered microglia function during chronic HIV-1 infection, especially given the interplay between HIV-1 infection and aging. It is also known that some pro-inflammatory cytokines are part of the unique secretory phenotype of cellular senescence, a cellular process not only known to occur during aging, but that can be at the origin of tissue/organismal aging. Moreover, microglia also express higher protein levels of cell cycle inhibitors and exhibit altered autophagy, both of which are integral parts of cellular senescence (Table 1), but not of activation. Thus, we propose that, although microglia senescence has not been fully characterized, it is likely an important part of the processes triggered in the CNS during chronic HIV-1 infection. Examining pathways implicated in the induction and consequences of cellular

senescence during HIV-1 infection could potentially lead to novel therapeutics for the treatment of HIV-1-associated end organ diseases, including HAND.

Chapter 2: Age-like phenotype in Microglia During HIV-1 Infection

This chapter is submitted as a paper for publication.

2.1 Introduction:

Although dementia associated with chronic HIV-1 infection has gradually become a rare clinical occurrence in the post combined antiretroviral treatment (cART) era, around half of all infected patients continue to develop HIV Associated Neurocognitive Disorders (HAND) to varying degrees (Chan and Brew 2014; Heaton et al. 2011; Sacktor et al. 2016). Alarmingly, the severity of HAND is expected to rise due to the aging of the HIV positive population. Studies show that even the mildest form of HAND—asymptomatic neurocognitive impairments (ANI) – could potentiate age-associated cognitive impairment resulting in the development of more serious neurocognitive deficits later in life as the patients age (Grant et al. 2014). Currently there are no therapy designed specifically for the treatment of HAND and more studies are needed to reveal mechanistic insights for the design and development of targeted, protective and adjunctive therapies that could more effectively ameliorate the neurocognitive impairments during chronic HIV-1 infection.

Microglia cells constitute a major target of productive HIV-1 infection in the CNS and could potentially serve as the cellular reservoir during chronic HIV-1 infection refractory to cART (Gonzalez-Scarano and Martin-Garcia 2005). A plethora of studies have demonstrated that microglia become activated during HIV-1 infection as evidenced by up-regulation of activation markers including HLA DR and release of pro-inflammatory cytokines such as TNFα, owing to both active viral replication and exposure to viral proteins (Flora et al. 2006; Garvey et al. 2013; Tatro et al. 2014; Walsh et al. 2014). It has been proposed that functional changes of microglia, particularly microglia activation during HIV-1 infection, might contribute to the development of HAND(Garvey et al. 2013). Furthermore, it is also known that microglia undergo aging associated functional changes and activation could be a component of the microglia aging program in both healthy subjects as well as patient with aging associated neurocognitive impairments

(Frank et al. 2006; Schuitemaker et al. 2012; Wong 2013). It was suggested that the activation of microglia could be an essential component of microglia function to maintain CNS homeostasis, however, persistent and unregulated microglia activation could result in dysfunctionally aged microglia with characteristics resembling cells that undergo senescence (Flanary and Streit 2004; Streit et al. 2004). Cellular senescence is known to be associated with loss of organ function associated with aging including the onset of Alzheimer's disease (Bhat et al. 2012). Evidence suggests that microglia could develop an age-like phenotype that resembles cellular senescence during the development of HAND. For instance, studies demonstrate elevated p53-p21 pathway, which is a characteristic marker of cellular senescence, in brain tissues of diseased HIV positive patients who developed HAND compared to neurocognitively normal patients (Garden et al. 2004; Jayadev et al. 2007).

To explore whether microglia undergo age-like process during HIV-1 infection, we examined various markers of aging associated cellular senescence including cell cycle inhibitors (p21, p53 and p16), DNA damage associated p53 binding protein 1 (53BP1) foci formation, senescence associated β-galactosidase (SA-β-gal) activity, and the development of Senescence Associated Secretory Phenotype (SASP) (Munoz-Espin and Serrano 2014). Additionally, since recent publications indicate that mitochondrial dysfunction is a crucial component of organismal aging, either as a cause or consequence to the onset of the aging process, we examined the production of mitochondrial reactive oxygen species (ROS) and mitochondrial electron transport chain (ETC) respiration (Wiley et al. 2016).

Our results indicate that HIV-1 infection could lead to the development of age-like phenotypes in human fetal microglia including elevated p21 expression, formation of 53BP1 foci, elevated SA-β-gal activity and the development of SASP. Associated with the onset of age-like phenotypes, we also detected elevated mitochondrial ROS

production as well as reduced basal ETC respiration, maximal ETC respiration and ATP-linked ETC respiration. Interestingly, we also observed that supernatant from age-like microglia induced elevated aging associated markers in naïve microglia cells, suggesting that components of SASP could initiate age-like program in microglia, which could amplify the detrimental effects of aging microglia during HIV-1 infection.

2.2 Materials and Methods

2.2.1 Cell Culture

Human fetal microglia (HFM) cells derived from fetal brain tissue (gestational age, 16-18 weeks) were provided by Temple Comprehensive NeuroAIDS Center Basic Science Core-1 in full compliance with National Institutes of Health and Temple University ethical guidelines. Cells are cultured at 37°C and 5% CO_2 in Microglia Growth Media (DMEM:F12, 15% FBS , 2 mM L-glutamine, 50mg/L Gentamicin, 5ug/mL Fungizone, 10mg/L Insulin, 10ug/L D-biotin, 10 mL NI supplement).

2.2.2 Flow Cytometry Analysis

HFM purified by shaking contain >96% Ionized Calcium Binding Adaptor Molecule 1 (Iba-1)-positive cells (Figure 2). Positivity of Ionized calcium-binding protein 1 (Iba1) and cell cycle profile of microglia cells were determined by FACSCalibur™ (BD Biosciences, San Jose, CA). To analyze Iba-1 level, cells were fixed and permeabilized and then incubated with FITC conjugated anti-Iba-1 antibody (Abcam ab 15691; clone: 1022-5; Isotype: IgG2b) at 2.5ul/10^6 cells or isotype control antibody at 2.5ul/10^6 cells for 30 min on ice. To analyze cell cycle, cells were harvested and fixed with cold absolute ethanol while vortexing followed by 1 hr incubation at 4°C. Post fixation, cells were incubated with 1 ml propidium iodide (PI) (Sigma, St. Louis, MO) staining solution containing 10ug/ml RNase A (Worthington Biochemicals, Lakewood, NJ) (Figure 3). The results were then analyzed with FlowJo. Mitochondrial ROS Analysis: For mitochondrial

ROS studies, cells were incubated with 5 μM MitoSox superoxide anion indicator (Molecular Probes, Carlsbad, CA) in microglia medium at 37°C in 5% CO_2 for 30 min, harvested in 2.5% trypsin–EDTA, re-suspended in 200 μL of complete growth medium, and analyzed immediately with a Guava Easy-Cyte Mini using the Guava Express Plus program (Millipore, Billerica, MA). Data is then analyzed using InCyte™ (Millipore, Billerica, MA) and mean fluorescence intensity is obtained for comparative analysis

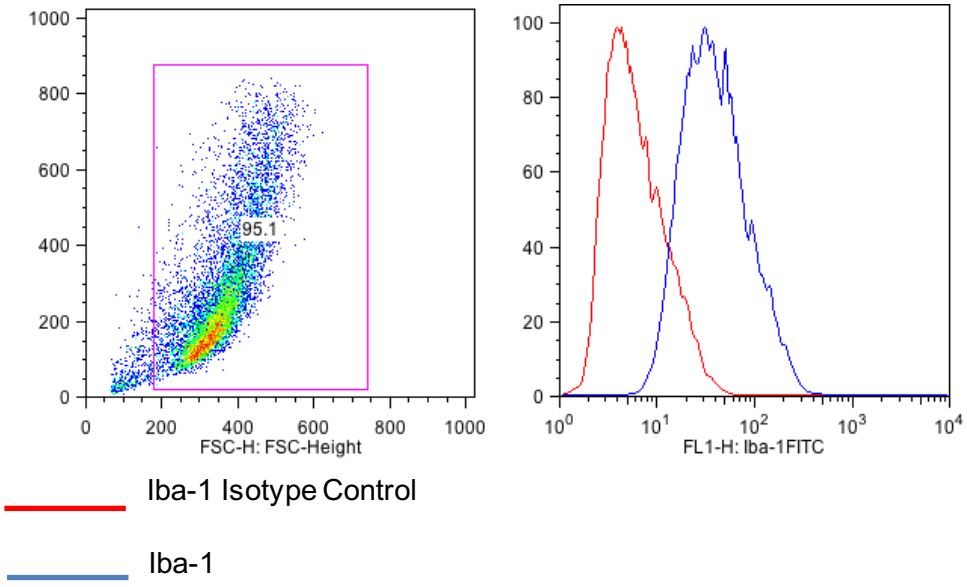

Figure 2. HFM is Positive for Iba-1 Staining. HFM is stained with macrophage/microglia marker Iba-1. Histogram is shown to the right. Red line represents isotype control for Iba-1 and blue line represents Iba-1 staining.

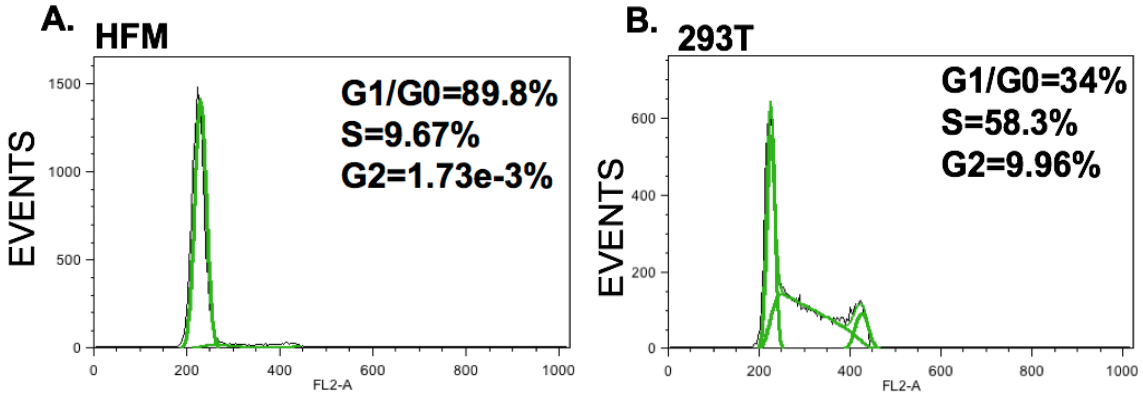

Figure 3. Cell Cycle Profile. HFM and 293T cells were fixed with ethanol, stained with PI and analyzed for DNA content. A. 89.8% of HFM were in G1/G0 phase, 9.67% of HFM were in S phase and 1.73 e^{-3} % HFM were in G2 phase. B. 34% of 293T cells were in G1/G0 phase, 58.3% of 293T were in S phase and 9.96 % 293T were in G2 phase.

2.2.3 Viral Production and Infection

To produce Env-pseudotyped, luciferase-reporter viruses, 293T cells were co-transfected using calcium phosphate precipitation (ProFection Mammalian Transfection System, Promega, Madison, WI) with VSV-G expression vector and with the Env-deficient, pNL4-3-luc+env- provirus. pNL4-3-luc+env- was developed by N. Landau by introducing a frame shift mutation in the env gene of pNL4-3-luc+ vector. VSV-G expression vector is utilized to facilitate cellular entry. For controls, 293T cells were transfected with either VSV-G or pNL4-3-luc+env- expression vectors alone. Culture supernatants containing the pseudotyped particles or control proteins were collected 48–72 hours after transfection, clarified by centrifugation, aliquoted and stored at -80°C until use. 1:2 dilution of viral stocks were used to infect target cells (HFM) at 37°C and 5% CO_2. To assess infectivity, at 6 days post-infection, infected cells were washed with PBS and lysed, and infectivity was measured by detecting luciferase activity (Luciferase Assay System, Promega) in a microplate luminometer (GloMax, Promega) (Figure 4). Multiplicity of infection (MOI) of 0.005 was used. MOI was assessed based on $TCID_{50}$ values. $TCID_{50}$ assays were conducted in HFM plated at 2*10^4 cells per well in a 96-well plate. Twelve 1:2 serial dilutions of viral stocks were performed in quadruplicates per dilution. Luciferase activities were used to determine $TCID_{50}$/mL using the Spearman and Kärber algorithm(Ramakrishnan 2016). MOI was then calculated using the following equation: $MOI = \frac{(0.7\ X\ \mathrm{TCID50}\ X\ volume\ of\ infection)}{Total\ number\ of\ target\ cells}$.

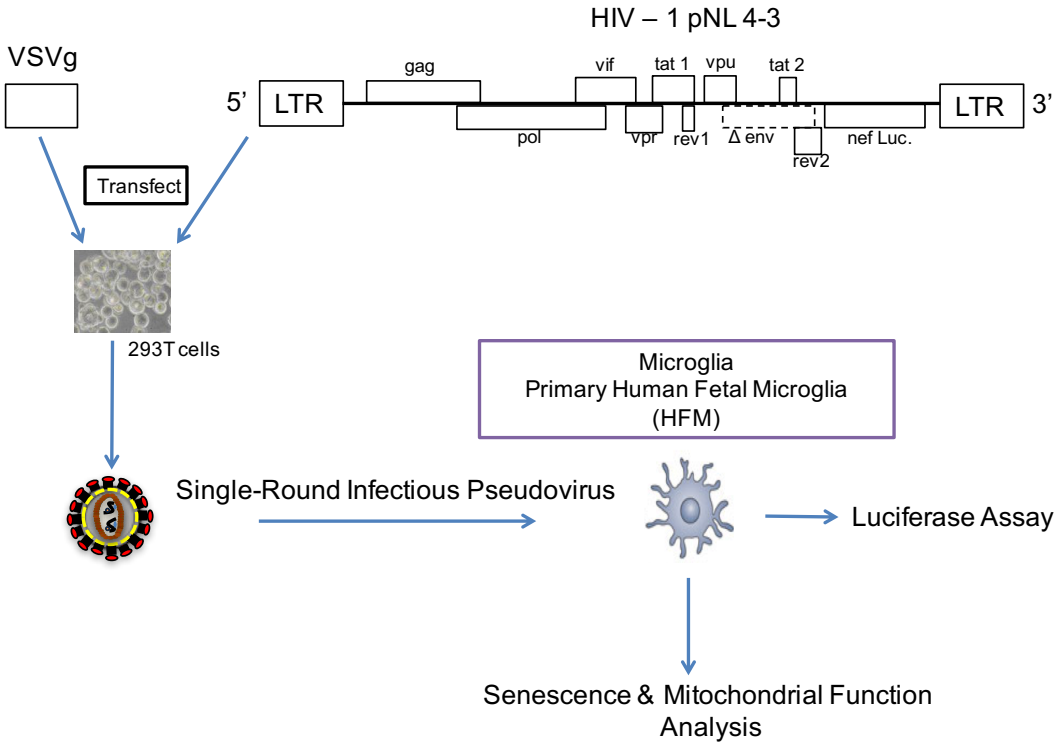

Figure 4. Flow-chart of Experimental Design.

2.2.4 Senescence-associated β-galactosidase activity

Senescence-associated beta-galactosidase (SA β-gal) activity was performed as previously described(Bitto et al. 2010). Briefly, following exposure to HIV-1 or transfection control supernatant, microglia were fixed in 2% formaldehyde/0.2% glutaraldehyde for 5 minutes and stained overnight for SA β-gal activity using X-gal as substrate. The percent of positive (blue) cells were counted. At least 200 cells were counted per condition, in triplicate.

2.2.5 Immunoblotting

Primary human fetal microglia were incubated with HIV-1 or control supernatant diluted in microglia media. After 4 days, media was changed. Cell lysate was collected 8 days post-treatment in RIPA buffer containing protease and phosphatase inhibitors. Western blot analysis was performed under standard conditions using 20 µg of total cell proteins and stained for specific proteins using anti-$p16^{INK4a}$ (1:200, BP Pharmingen, San Jose, CA), anti-p21(1:200, H-164, Santa Cruz Biotechnology), anti-p53 (1:500, Ab-6, Calbiochem, Billerica, MA) antibodies as well as anti-β-actin (1:500, Sigma Aldrich; St. Louis, MO) or anti-GAPDH (1:500, Calbiochem, Billerica, MA)) antibodies as loading controls.

2.2.6 Immunofluorescence

Cells were plated in Falcon Culture Slides (Corning) at 10^4 cells per chamber post treatment. Cells were first fixed for 10 minutes with 4% paraformaldehyde at room temperature (RT) and then permeabilized for 15 minutes with 0.2% TritonX-100. Slides were blocked in 5% normal goat serum, 0.1% PBS-BSA for 2 hours, then incubated overnight in a humidified chamber at 4ºC with primary rabbit antibody anti- 53BP1(Novus Biologicals, Littleton, Colorado) diluted at 1: 500 in PBS 0.1% BSA. Slides were washed 3 times for 5 minutes in 0.1% BSA and 0.1% Triton X-100, then incubated for 1 hour in

the dark at room temperature with the secondary antibody goat anti-rabbit Alexa Fluor 555 (Invitrogen; Carlsbad, CA). After 3×5 minute washes in 1X PBS with 0.1% BSA and 0.1% Triton X-100, slides were incubated with DAPI, and mounted with Vectashield Mounting Medium (Vector Laboratories, Inc.; Burlingame, CA). Cells were visualized using an Olympus BX61 fluorescence microscope coupled with a Hamamatsu ORCA-ER camera and using Slide Book 4 software version 4.0.1.44 (Intelligent Innovations, Inc.; Denver, CO).

2.2.7 Mitochondrial Electron Transport Chain (ETC) Function

For real-time analysis of Oxygen Consumption Rate (OCR), microglia were analyzed with an XF-96 Extracellular Flux Analyzer (Seahorse Bioscience, North Billerica, MA). Three consecutive measurements were obtained under basal conditions and after the sequential addition of 5 μM oligomycin, to inhibit mitochondrial ATP synthase; 4 μM FCCP (fluoro-carbonyl cyanide phenylhydrazone), a protonophore that uncouples ATP synthesis from proton gradient generated the electron-transport chain through respiration; and 1.8 μM rotenone plus antimycin A, which inhibit complexes I and III respectively of the electron transport chain (Seahorse Bioscience, North Billerica, MA). In this assay, basal oxygen consumption can be established as measured of OCR in the absence of drugs. Reduced OCR after the addition of oligomycin and of rotenone and antimycin indicate that cells are consuming less oxygen in response to reduced mitochondrial oxidative phosphorylation. Maximal OCR occurs after the addition of FCCP due to cells' attempt to maintain a proton gradient across the inner mitochondrial membrane by increasing the consumption of oxygen (Huang et al. 2014). The oligomycin sensitive portion of mitochondrial respiration represents ATP-linked OCR and oligomycin insensitive portion represents proton leak (Keuper et al 2014) (Figure 5). The OCR is represented as absolute values (pMoles/min) normalized by the number of cel

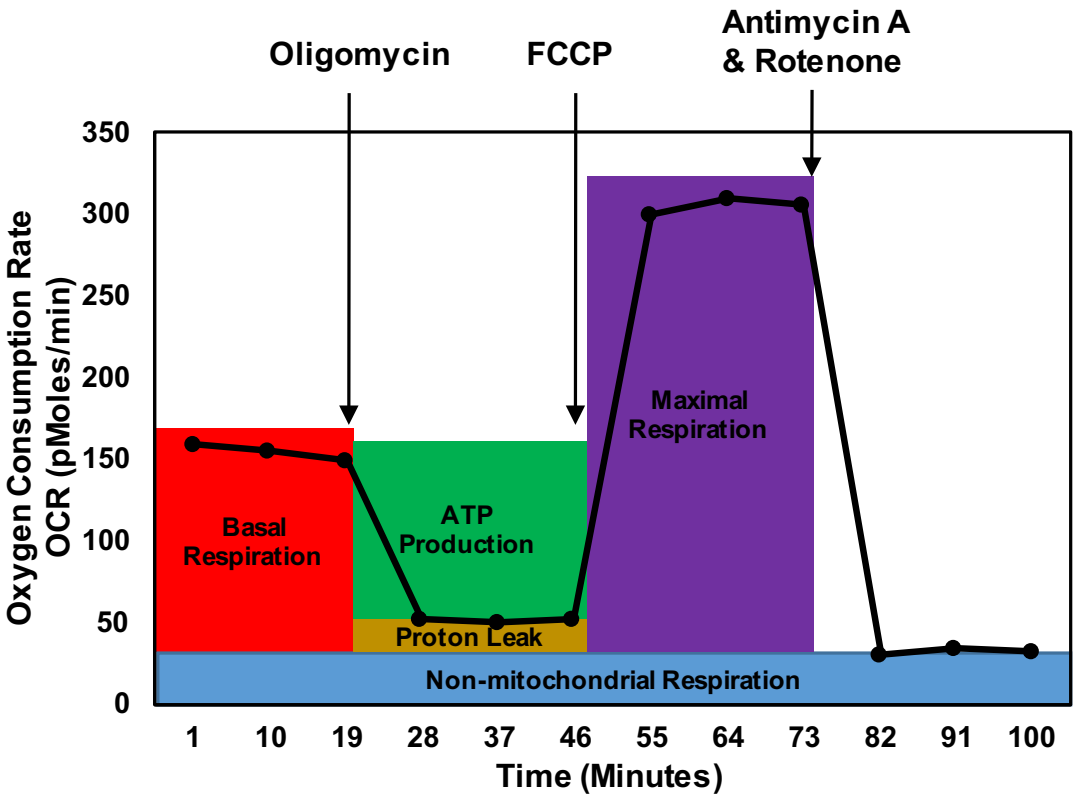

Figure 5. Mitochondrial Respiration Analysis. Parameters of mitochondrial functions include basal respiration, ATP linked respiration, maximal respiration and spare respiration can be calculated based on oxygen consumption rates upon treatments with oligomycin, FCCP and Antimycin A & Rotenone.

2.2.8 Analysis of Cytokines Secreted by Human Microglia Cells

Control and infected HFM were washed twice with PBS and supernatant was replaced with serum-free MCDB105 media 4 days post treatment (Sigma Aldrich; St. Louis, MO). After 24-hour incubation, conditioned media were collected and cells were trypsinized and counted to determine cell number for normalization. Human cytokine antibody array (RayBiotech, Inc.; Norcross, GA) was used to detect cytokines present in the collected media according to product manual. The intensity of the signal on the array membranes was quantified by densitometry using ImageJ (NIH; Bethesda, Maryland) and then normalized by cell number. Supernatant from three cases were analyzed to confirm the secretory profile. To validate selected cytokines that are altered in all three cases, enzyme-linked immunosorbent assay (ELISA) for Interleukin 8 (IL8), Interleukin 6 (IL6), Vascular Endothelial Growth Factor A (VEGF-A) and Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF) (eBioscience; San Diego, CA) were performed. Results were normalized to the number of cells and represented in ng/ml per 10^6 cells.

2.2.9 Supernatant Transfer Assay

To examine whether components of the microglia supernatant post infection affect innocent bystander cells, supernatant transfer assays were performed. Specifically, supernatant from infected, infected and control cells were collected four days post infection. treatment naïve microglia cells were incubated with supernatant diluted with microglia media at 1:3. One day post treatment, cell lysates were collected and examined for protein expressions of p21 and rabbit anti caveolin-1 (1:300, Cell Signaling; Danvers, MA).

2.2.10 Nucleoside (dNTPs) Treatment of Cells

Cell were treated with 250 nM of nucleoside cocktails (gift from Dr. Rugang Zhang, Wistar Institute Philadelphia, PA) at the same time as infection and replenished every four days.

2.2.11 Viability Staining

Cells were trypsinized and 4*10^4 cells were stained with ViaCount Reagent (Millipore, Billerica, MA) and analyzed with Guava ViaCount software (Millipore, Billerica, MA) Percentage of nucleated and viable cells were graphed. Heat killed cells were used as positive control.

2.2.12 Statistical Analysis

Nonparametric analyses Paired Wilcoxon Signed Ranks Test (2-tailed) were performed with SPSS (IBM, NY). p values that indicate statistical significance or trends approaching significance are annotated on the graphs. All p values are presented in Appendix A.

2.3 Results:

2.3.1 Development of Age-like Phenotype in Microglia During HIV-1 Infectio

To determine whether microglia develop senescent phenotypes during HIV-1 infection, we examined various senescence markers including β-gal activity, expression of cell cycle regulators including p21, p53 and p16 as well as 53BP1 foci formation. Primary human fetal microglia were incubated with VSVg pseudotyped HIV-$1_{pNL43,}$ fresh media and transfection control containing VSVg or pNL43 for 8 days. Luciferase activities were measured to confirm infection (Fig. 27)**.** Cell cultures incubated with VSVg-pNL43 infectious virions have luciferase light units around 100000 in log scale compared to cells incubated with control supernatants (uninfected, VSVg and pNL43) with luciferase light units around 90 in log scale indicating successful infection in VSVg-pNL43 treated cells but not control treated cells.

To assess the development of SA β-gal, additional cells were stained for β-gal activity and images were taken and analyzed using ImageJ as well as CellProfiler (Figure 6). Numbers of cells positive for β-gal activity as indicated by blue color were counted. Samples infected with VSVg pseudotyped HIV-1_{pNL43} contained significantly elevated percentage of positive cells for SA β-gal (25.1%) compared to all controls (untreated: 10.3%, VSVg 10.9% and pNL43: 14.6%) (Figure 7). Treatment with Neocarzinostatin, a DNA damage agent used as inducer of senescence, results in the highest percentages of cells positive for SA β-gal (32.3%) (Hewitt et al. 2012). The elevated incidence of SA β-gal positivity post HIV-1 infection suggests that HIV-1 infection, either the process of viral infection or exposure to soluble factors associated with viral infection, results in the development of cellular senescence in microglia cells.

To perform a more comprehensive analysis of the senescence program, protein expression of cell cycle regulators p16, p53 and p21, were analyzed upon incubation

with VSVg pseudotyped HIV-1_{pNL43} or controls since elevated levels of these markers have been observed during the development of cellular senescence in several cell types including brain cells (Campisi and d'Adda di Fagagna 2007). Post treatment, lysates were extracted to assess the protein level of p53, p16 and p21 using Immunoblot (Figure 8). Compared to control treated cells, HIV-1 infected microglia exhibit significantly elevated p21 expression (3.14 fold increase over untreated controls) compared to VSVg treated (1.14 fold increased over untreated controls) (Figure 9). Although infected microglia also express higher levels of p21 compared to pNL43 treated controls (0.95 fold increase over untreated), the difference is not statistically significant. No statistically significant change is observed in p53 and p16 levels. This suggests that HIV-1 infection induced microglia senescence likely occur through the p21 pathway and not the p16 mediated signaling pathway. This is further strengthened by the observation of elevated 53BP1 foci (Figure 10). 53BP1 foci is constitutively detected in untreated cells suggesting basal level of on-going DNA damage response to maintain genomic stability as a house-keeping function. The prevalence of 53BP1 foci formation is significantly elevated in infected cultures. 53BP1 foci formation indicates the activation of DNA damage response post infection upstream of p21 pathway (Lossaint et al. 2011). Activation of DNA damage repair has been previously reported to be a critical step required for efficient HIV-1 infection, which could contributes to the onset of cellular senescence since DNA damage response including the formation of 53BP1 foci, is often observed in senescent cells (d'Adda di Fagagna et al. 2003; Roshal et al. 2003). Overall, elevated SA β-gal activity, increased p21 expression and formation of 53BP1 foci all support the development of senescence program in microglia during HIV-1 infection.

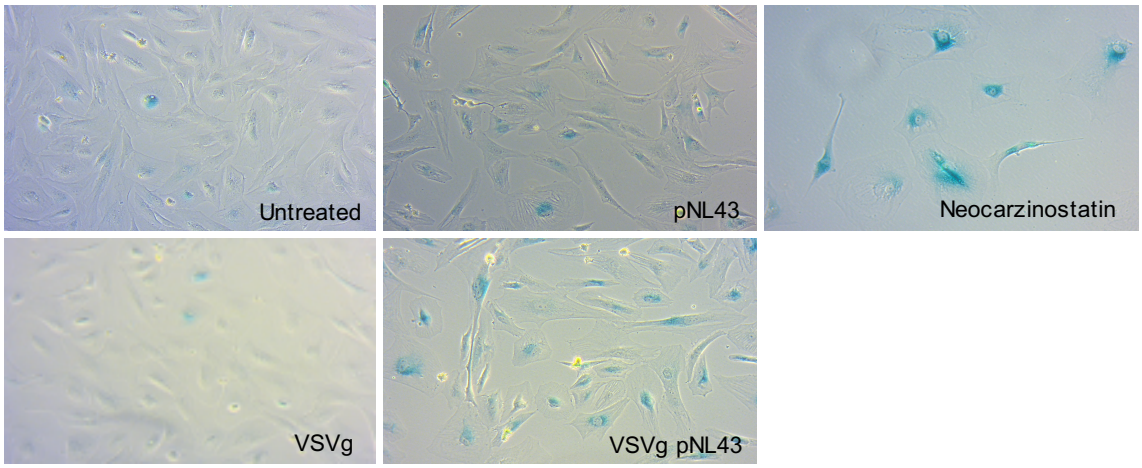

Figure 6. SA-β-gal Staining of HFM Post HIV-1 Infection. Representative pictures and of Senescence Associated β-gal (SA-β-gal) positive cells (blue). Controls and infected cells were fixed and stained to detect β-gal activities. More SA-β-gal positive cells are detected in VSVg pNL43 and neocarzinostatin treated cells indicating elevated β-gal activities compared to all controls.

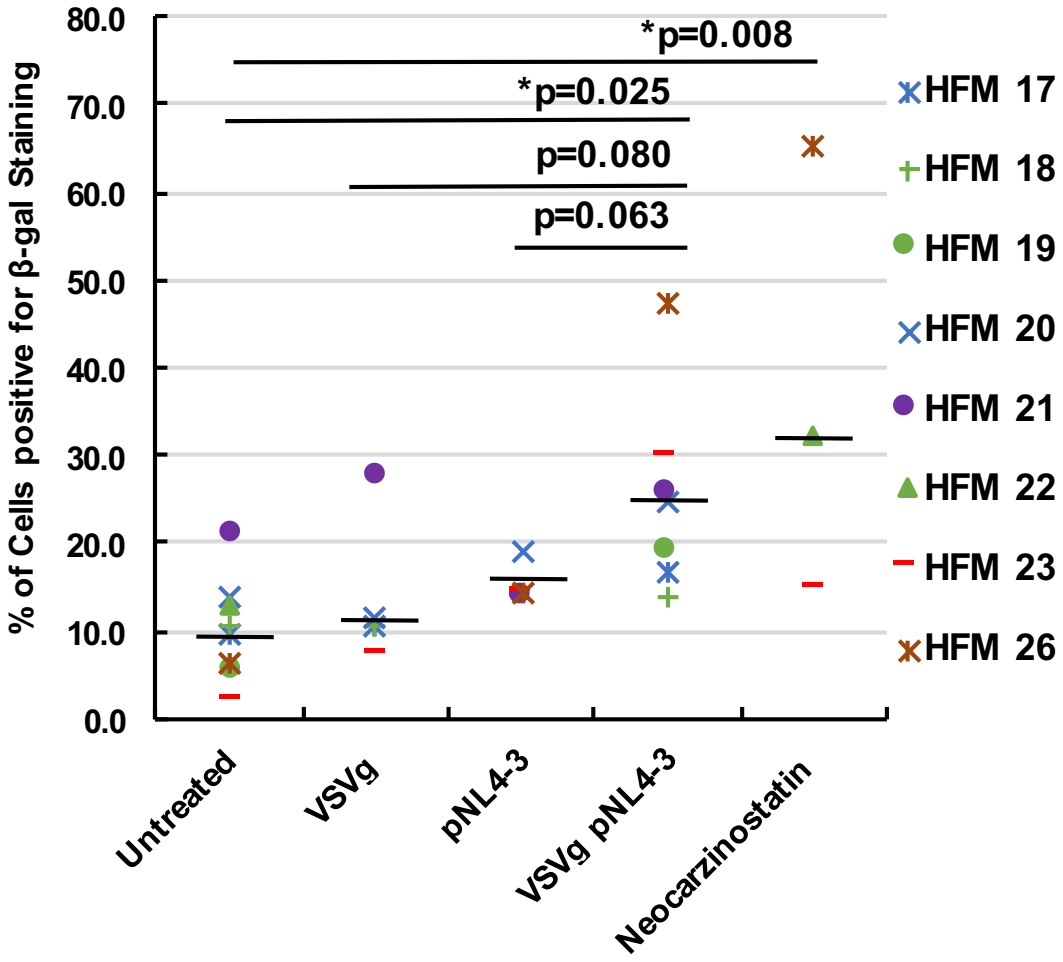

Figure 7. Increased percentages of cells positive for Senescence Associated β-gal (SA- β-gal) post HIV-1 infection. Total of 8 cases were used. Untreated: n=8, pNL43: n=4, VSVg; n=5, VSVg pNL43, n=7 and Neocarzinostatin, n=3. Black bars represent the medians for each treatment group (Untreated =10.3; VSVg = 10.9; pNL43 = 14.6; VSVg pNL43 = 25.1; Neocarzinostatin = 32.3). Nonparametric analysis (2-tailed) was performed. p values are annotated on the graph. * indicates statistical significance.

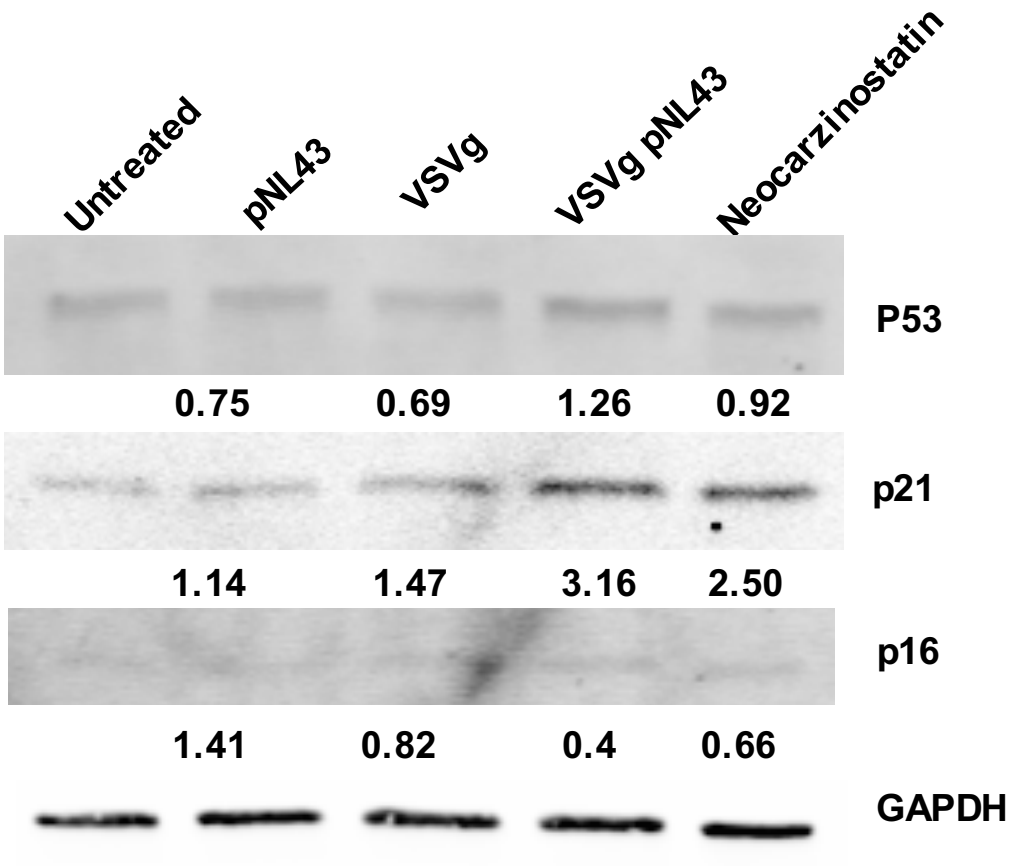

Figure 8. Immunoblot of cell cycle regulators. p53, p21, p16 and GAPDH proteins levels were detected with antibodies for untreated, pNL43, VSVg, VSVg pNL43 and Neocarzinostatin treated samples. Fold change of densitometry values for each treatment over untreated is annotated below the corresponding lanes.

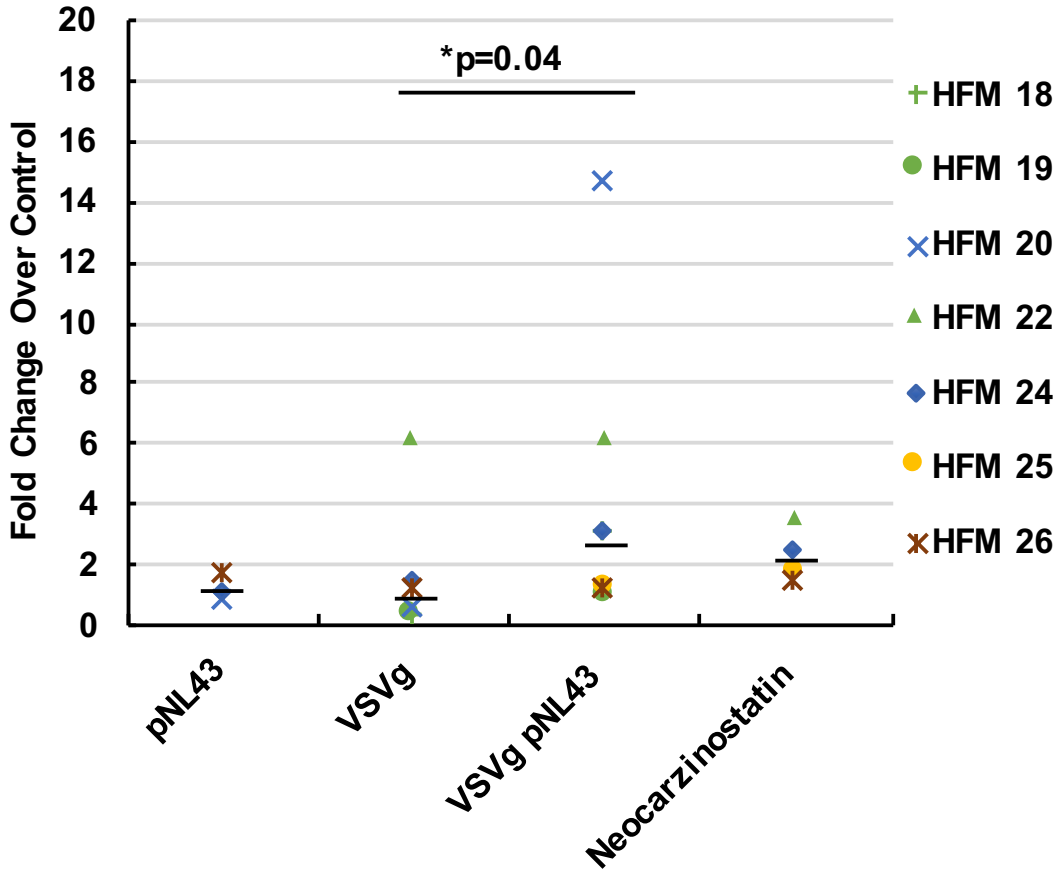

Figure 9. Elevated p21 protein expression post HIV-1 Infection. Densitometry values normalized by untreated cells for each treatment are graphed. Total of 7 cases were analyzed. Untreated: n=7, VSVg: n= 6, $pNL_{4\text{-}3}$: n= 3, VSVg $pNL_{4\text{-}3}$: n=7, Neocarzinostatin, n=4. Black bars represent the medians for each treatment group (pNL43 = 1.14; VSVg = 0.95; VSVg pNL43 = 2.15). Nonparametric analysis (2-tailed) was performed. p values are annotated on the graph. * indicates statistical significance.

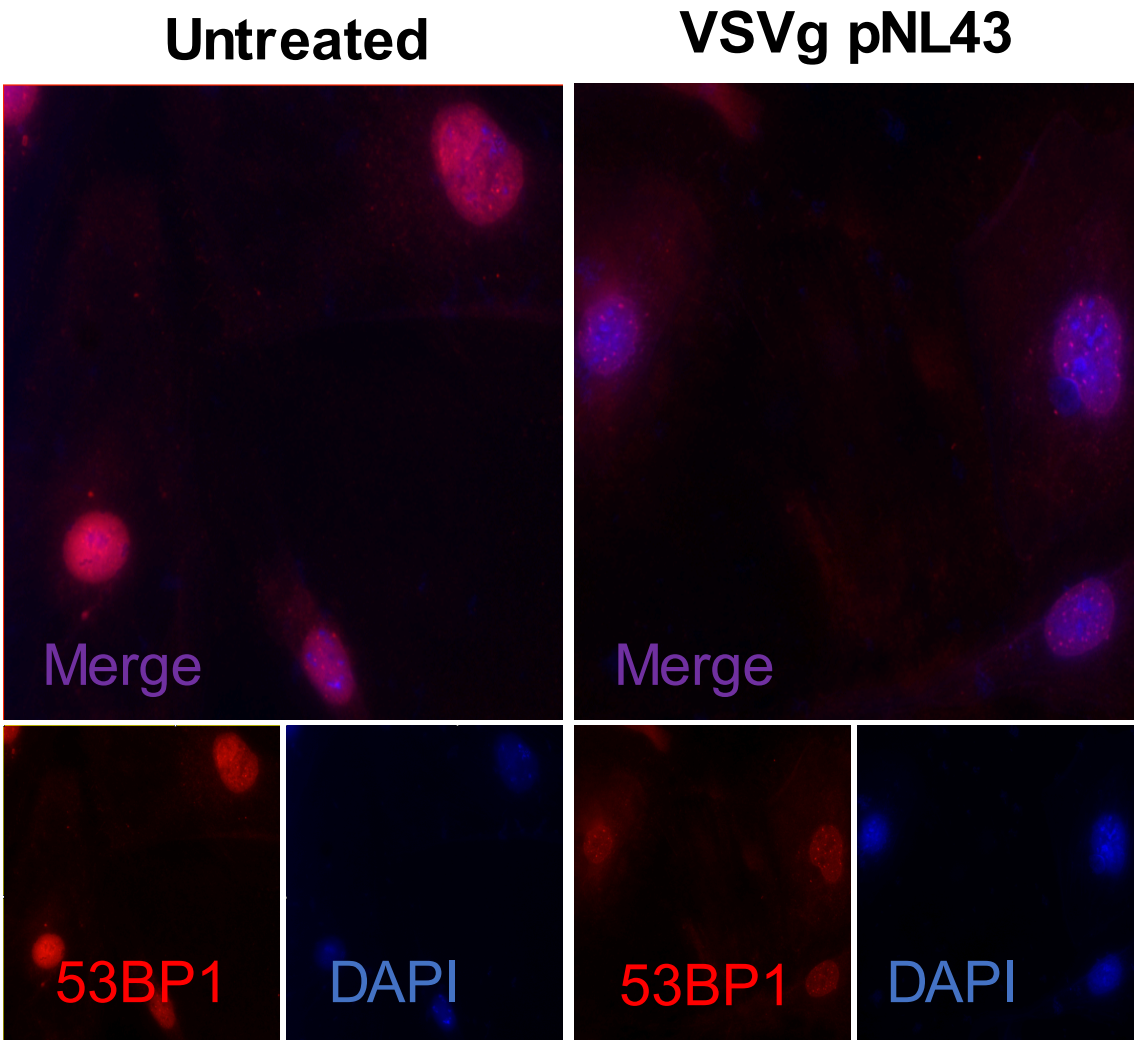

Figure 10. Increased 53BP1 DNA damage foci formation post HIV-1 infection. Immunofluorescence staining for DNA damage foci 53BP1 (red) and nuclei marker DAPI (Blue) are shown.

2.3.2 Elevated Mitochondrial Reactive Oxygen Species (ROS) Production is Associated with Microglial Age-like Phenotype

Mitochondrial ROS associated oxidative damage contributes to the development of various neurodegenerative diseases and therapies targeting ROS generation in mitochondrial holds great promises in protecting neuronal integrity(Lin and Beal 2006). Recent reports strongly suggest an association between mitochondrial dysfunction and the development of senescence in various cell types. To explore whether mitochondrial integrity in microglia during HIV-1 infection is altered in the context of microglial aging, we measured mitochondrial ROS production post HIV-1 infection. Primary human fetal microglia were infected with HIV-1 and then incubated with MitoSOX, a dye specific for mitochondrial ROS (Mukhopadhyay et al. 2007; Nacarelli et al. 2014). HIV-1 infected cells demonstrated significantly elevated mean fluorescence intensity (MFI) (median of 53) post staining compared to untreated cells (median of 26) suggesting elevated accumulation of ROS in mitochondria (Figure 11&12). Although infected cells also demonstrate higher MFI compared to VSVg (median of 38) and pNL43 (median of 47.8) control treated cells, the differences are not statistically significant, suggesting that increased mitochondrial ROS is not specific the HIV-1 infection process but a general stress response to external stimuli.

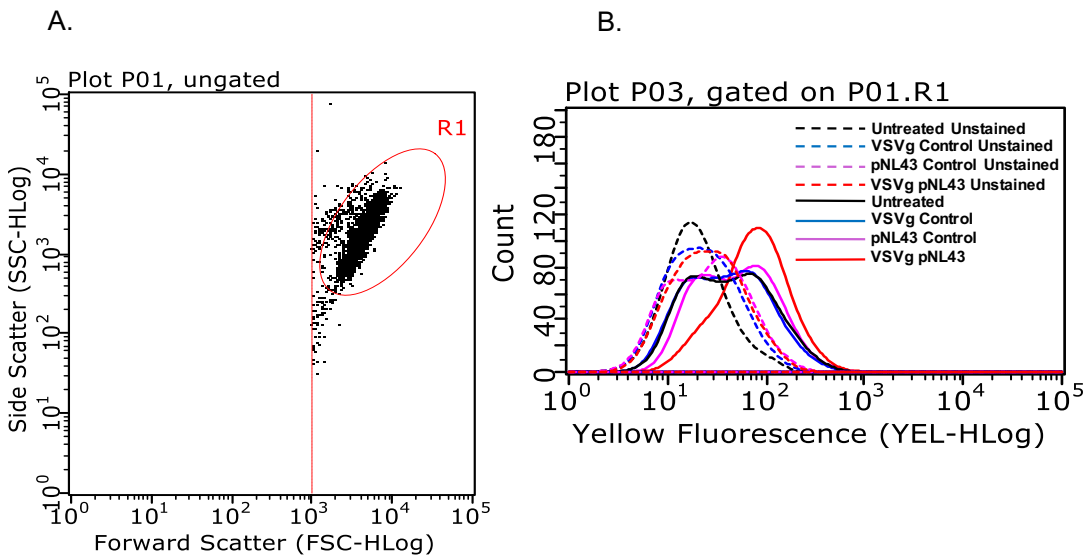

Figure 11. Flow-cytometry analysis of Mitochondrial ROS. Flow-cytometry was used to analyze MitoSOX staining intensity. A. Scatter plot of microglia demonstrating gating strategy. B. Histograms of fluorescence intensity for MitoSOX in control and infected samples. Dotted (unstained controls) and solid lines (stained samples) are color-coded to represent treatment groups as annotated in the legend.

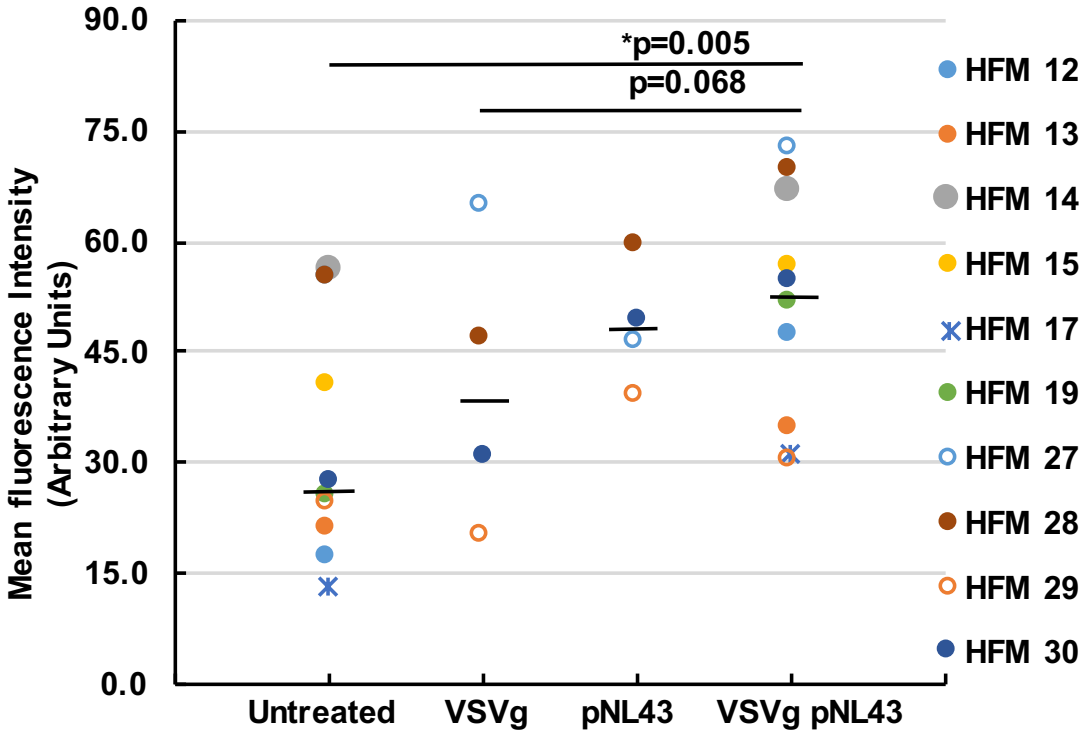

Figure 12. Elevated Mitochondrial ROS Production post HIV-1 Infection. Mean MitoSOX fluorescence intensity for control and infected cells post staining controlled for auto-fluorescence. Total of 6 cases were analyzed. Black bars represent the medians for each treatment group (Untreated = 26; VSVg = 38; pNL43 = 47.8; VSVg pNL43 = 53). Nonparametric statistic analysis was performed and p values are annotated on the graphs. * indicates statistical significance.

2.3.3 HIV-1 Infection Alters Mitochondrial Respiration

Since majority of mitochondrial ROS is generated by the electron transport chain (ETC), elevated mitochondrial ROS suggest an altered ETC homeostasis during HIV-1 infection. Mitochondrial ETC function was assessed using Seahorse Extracellular Flux (XF) Analyzer(Gallardo et al. 2014). Oxygen consumption rates (OCR) were measured in real time under basal condition and when challenged with inhibitors of various electron transport chain complexes including oligomycin, FCCP, and Antimycine A/Rotenone. The OCR of infected microglia was compared to that of control treated microglia (Figure 13). Basal respiration, ATP-linked respiration, maximal respiration, proton leak, and spare respiration were calculated based on OCRs. Figure 13 shows that the non-mitochondrial associated respiration is reduced to similar levels for infected as well as VSVg and pNL43 control treated cells suggesting that supernatant of transfected 293T cells inhibits cellular respiration that is not dependent on mitochondria. Basal respiration in VSVg-pNL43 infected cells (median of 38.5) is significantly lower than VSVg controls (median of 92) and lower than pNL43 controls (median of 99) (Figure 14). ATP-linked respiration in VSVg-pNL43 infected cells (median of 11) is also significantly lower than untreated (median of 23) and VSVg (median of 15) controls (Figure 15). Maximal respiration is significantly lower in VSVg-pNL43 infected cells (median of 59.8) compared to VSVg treated control cells (median of 87.7) as well (Figure 16). Proton leak and spare respiration were not significantly different between the controls and infected HFMs (Figure 17&18). Reduced basal, ATP-linked and maximal respiration in VSVg-pNL43 treated HFM compared to controls suggest impaired mitochondrial function as well as mitochondrial-linked cellular metabolism in HIV-1 infected microglia cells.

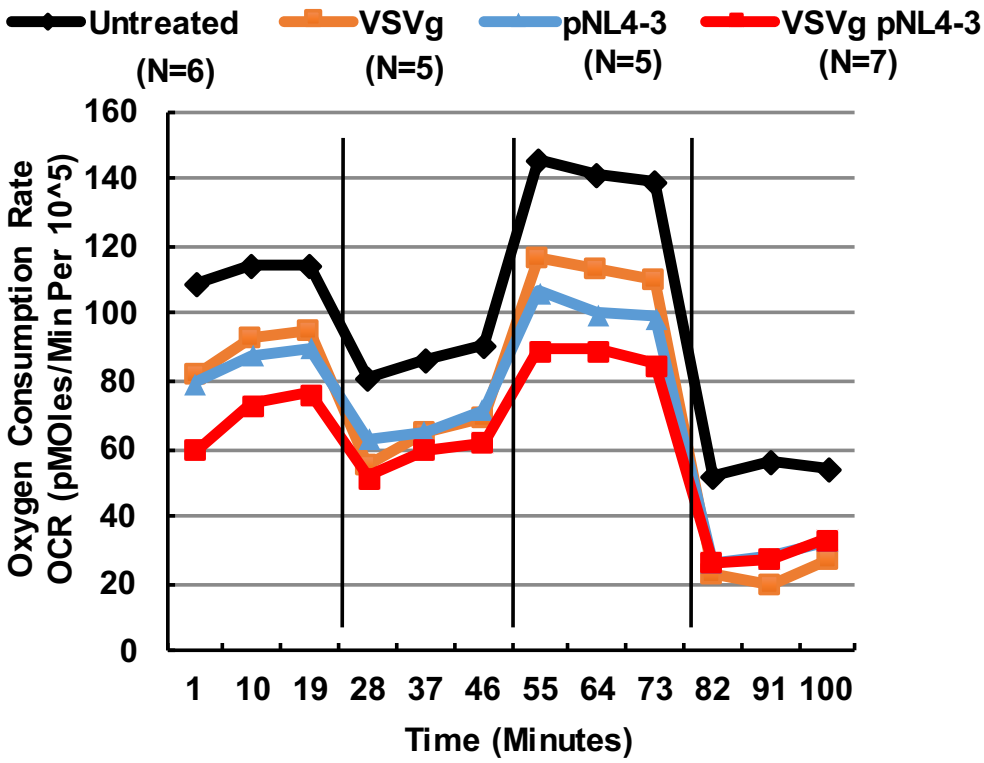

Figure 13. Mitochondrial ETC Respiration Graphs. Oxygen consumption rate normalized by cell number (pMoles/min per 10^5 cells) is graphed as a function of time (minutes). First black line indicates addition of 5 μM oligomycin, second black line indicates addition of 4 μM FCCP (fluoro-carbonyl cyanide phenylhydrazone), and third black like indicates addition of 1.8 μM rotenone plus antimycin. Data points represent averages of multiple cases as indicated in the legend.

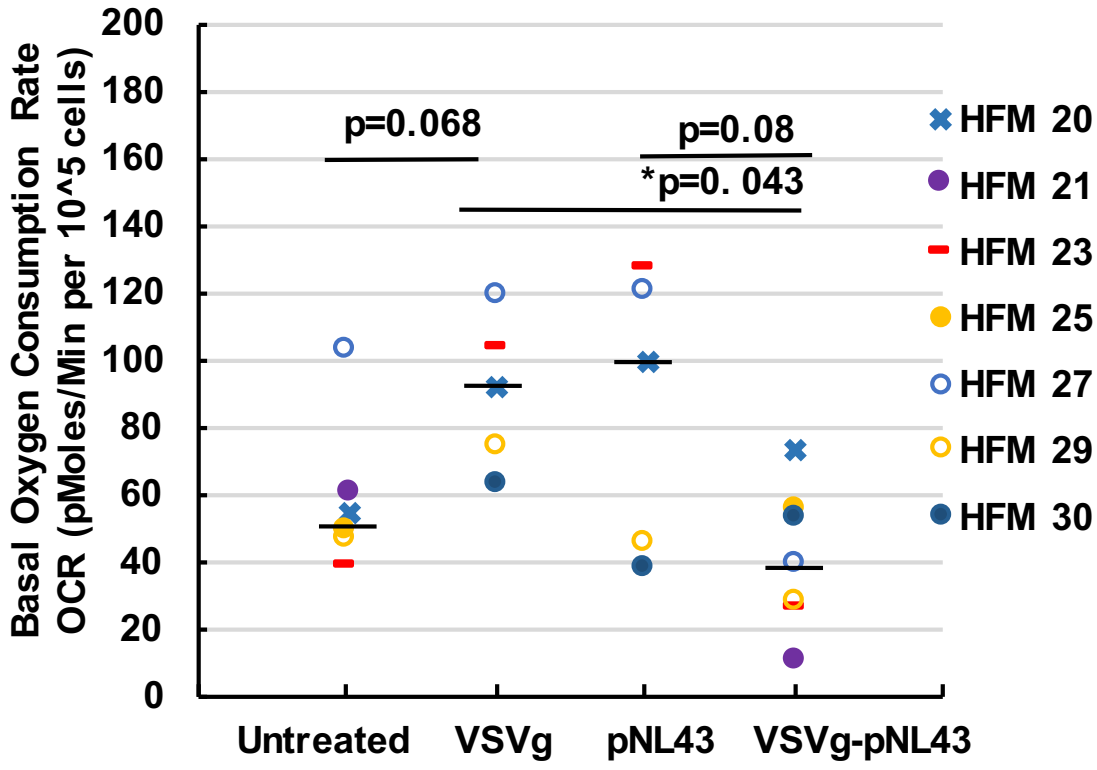

Figure 14. Basal ETC Respiration. Basal respirations are calculated and graphed. Medians for basal respirations are: Untreated = 51; VSVg = 92; pNL43 = 99; VSVg pNL43 = 38.5. Nonparametric statistic analysis was performed and p values are annotated on the graphs.* indicates significance.

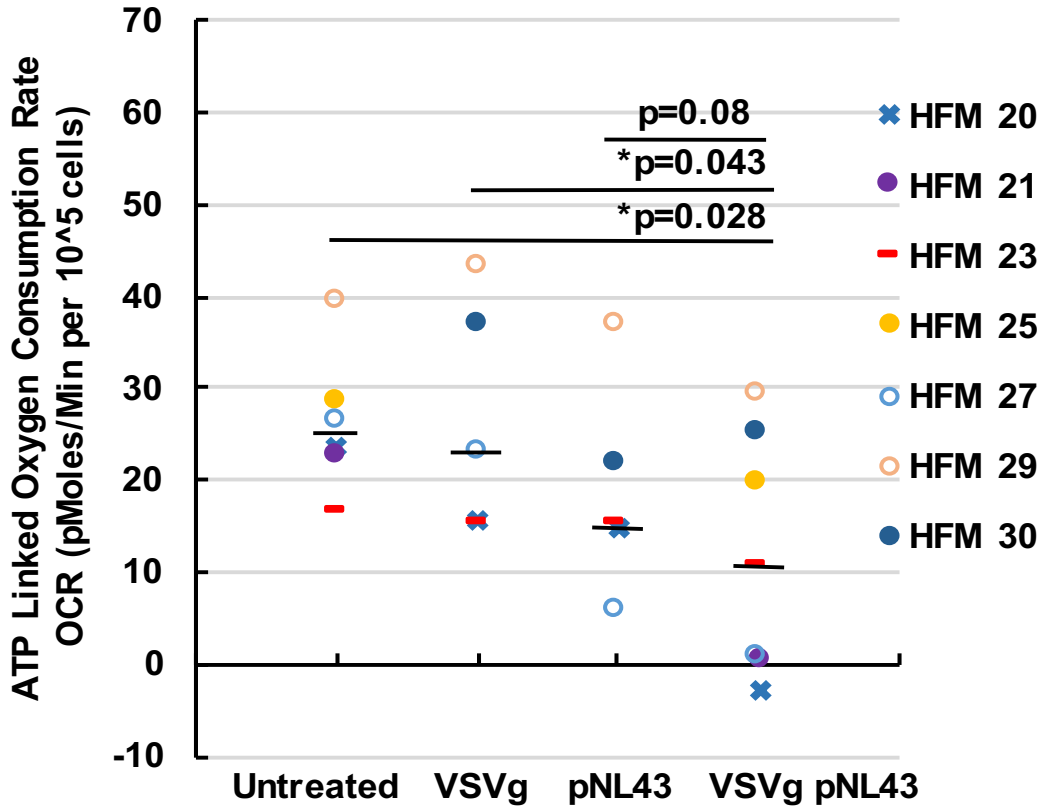

Figure 15. ATP linked ETC Respiration. ATP linked respirations are calculated and graphed. Medians for ATP-linked respirations are: Untreated = 25; VSVg = 23; pNL43 = 15; VSVg pNL43 = 11. Nonparametric statistic analysis was performed and p values are annotated on the graphs. * indicates significance.

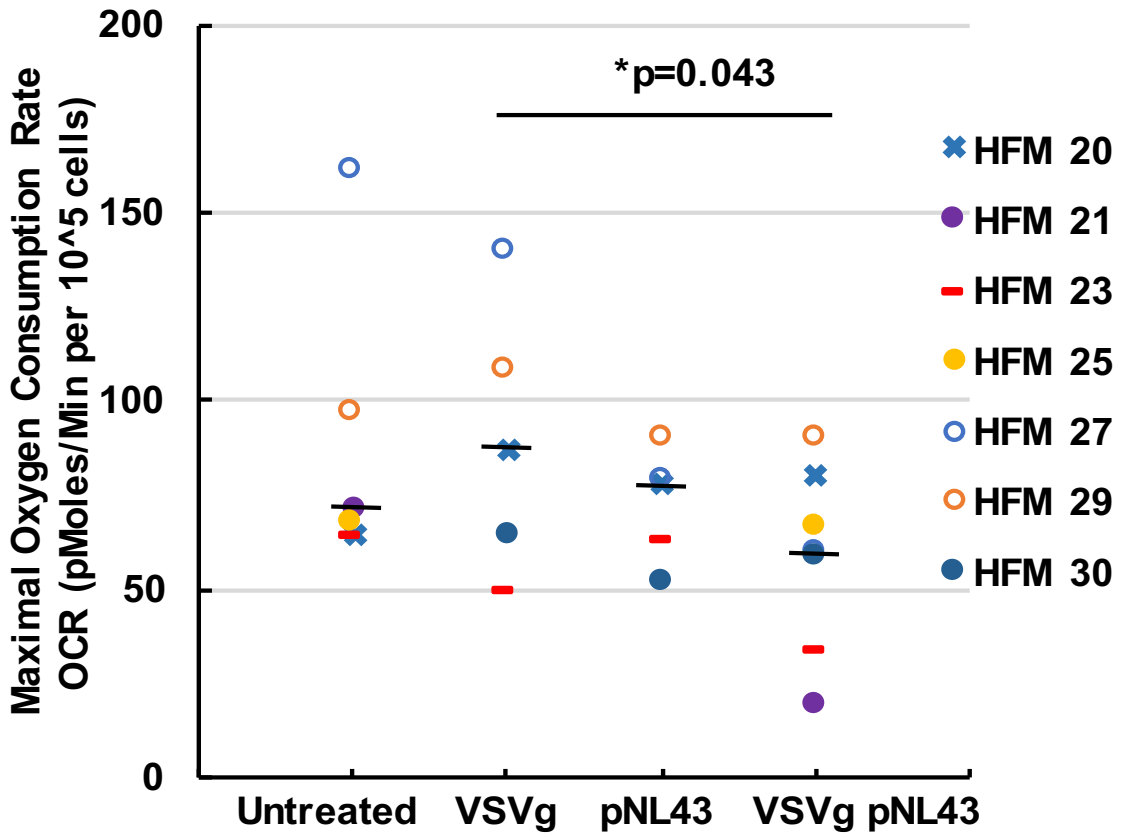

Figure 16. Maximal ETC Respiration. Maximal respirations are calculated and graphed. Medians for maximal respirations are: Untreated = 69.6; VSVg = 87.7; pNL43 = 78.1; VSVg pNL43 = 59.8. Nonparametric statistic analysis was performed and p values are annotated on the graphs. * indicates significance.

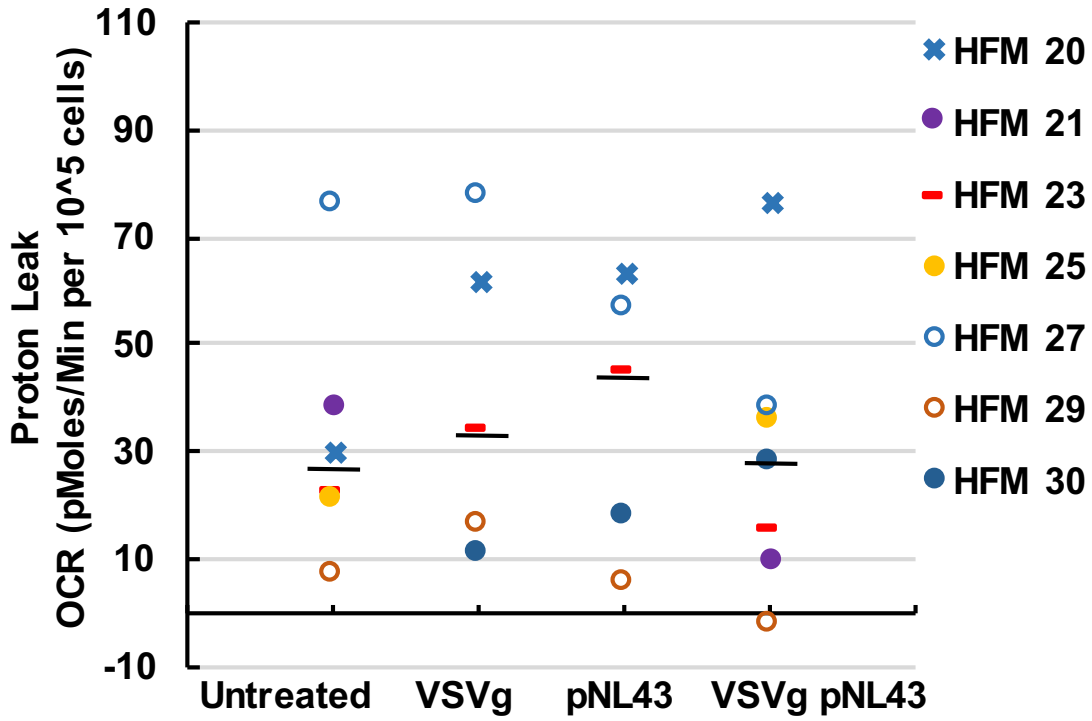

Figure 17. Proton-leak of ETC. Maximal respirations are calculated and graphed. Medians for proton leak are: Untreated = 26.3; VSVg = 334; pNL43 = 45.2; VSVg pNL43 = 27.5. Nonparametric statistic analysis was performed.

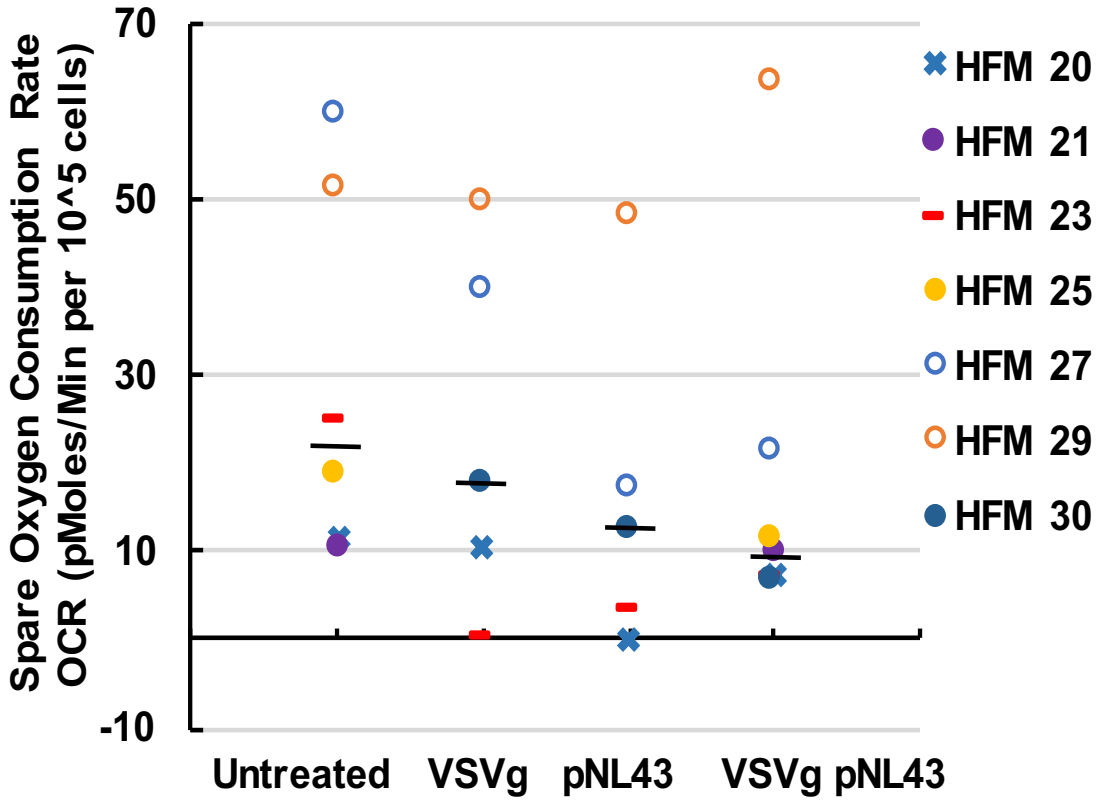

Figure 18. Spare ETC Respiration. Maximal respirations are calculated and graphed. Medians for spare respirations are: Untreated = 21.7; VSVg = 17.4; pNL43 = 12.5; VSVg pNL43 = 9.8. Nonparametric statistic analysis was performed.

2.3.4 Senescence Associated Secretory Phenotype Post Infection

Senescent cells develop a unique secretory phenotype mediated by DNA damage response, which both serves as a marker of cellular senescence and also plays an important role in the propagation of functional changes associated with senescence (Rodier et al. 2009). To examine the global cytokine profile secreted by microglia during HIV-1 infection, supernatants were analyzed using antibody array membranes (Figure 19). Thirteen cytokines including IL-6, IL-8, Vascular Endothelial Growth Factor (VEGF)-A and Granulocyte-macrophage colony-stimulating factor (GM-CSF), which are all classically associated with the development of cellular senescence (Coppe et al. 2010), were detected at elevated levels in the supernatant post infection compared to supernatant from untreated cells (Figure 20). Some cytokines including IL-6 and IL8 were present at basal conditions and secreted at much higher levels post infection. Selected cytokines such as VEGF-A and GM-CSF were absent in untreated cells but secreted at detectable albeit low levels post infection, which could also be physiologically significant (Figure 20). To quantitatively confirm the levels of secreted cytokines, we measured protein concentrations of secreted IL8, IL-6, VEGF-A and GM-CSF using ELISAs. In seven cases, IL8 levels were consistently elevated post infection (median: 108 pg/ml per $10^{\wedge 6}$ cells) compared to all controls (Figure 21). IL6 levels were also significantly elevated post infection (median: 470.87 pg/ml per $10^{\wedge 6}$ cells) compared to untreated (median: 64.04 pg/ml per $10^{\wedge 6}$ cells and pNL43 (median: 284 pg/ml per $10^{\wedge 6}$ cells) control cells. The median IL6 level of infected cells was also higher than that of VSVg (median: 284.01 pg/ml per $10^{\wedge 6}$ cells) control cells, approaching significance (Figure 22). VEGF-A (Figure 23) and GM-CSF (Figure 24) levels were not significantly altered post treatments.

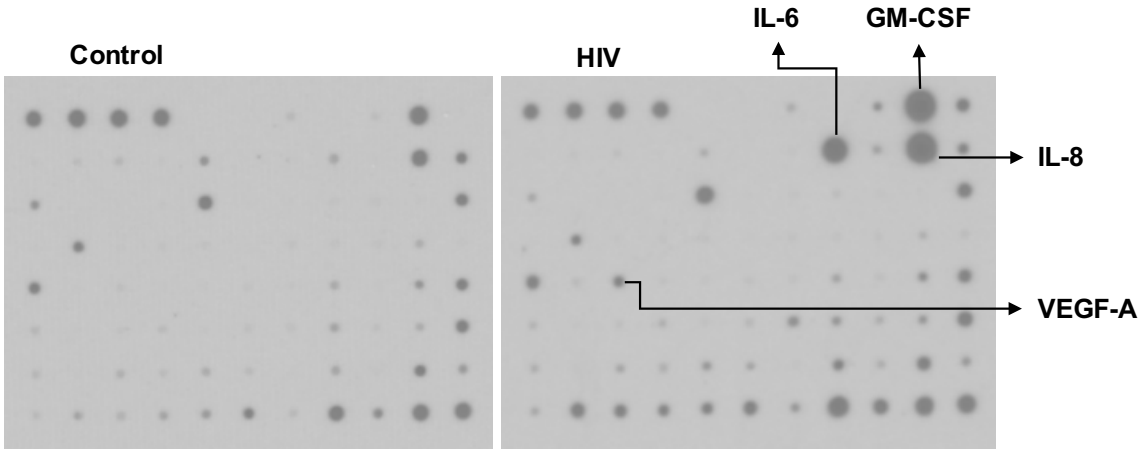

Figure 19. Antibody Array Immunoblots for Secreted Cytokines. Cytokine secretion profiles of untreated and HIV-1 infected samples demonstrated using antibody arrays. Three pairs were analyzed and result of one representative pair is shown. Arrows point to the cytokines that are evaluated with ELISAs (IL6, IL8, GM-CSF and VEGF-A). Representative images shown were obtained with supernatant from HFM 20.

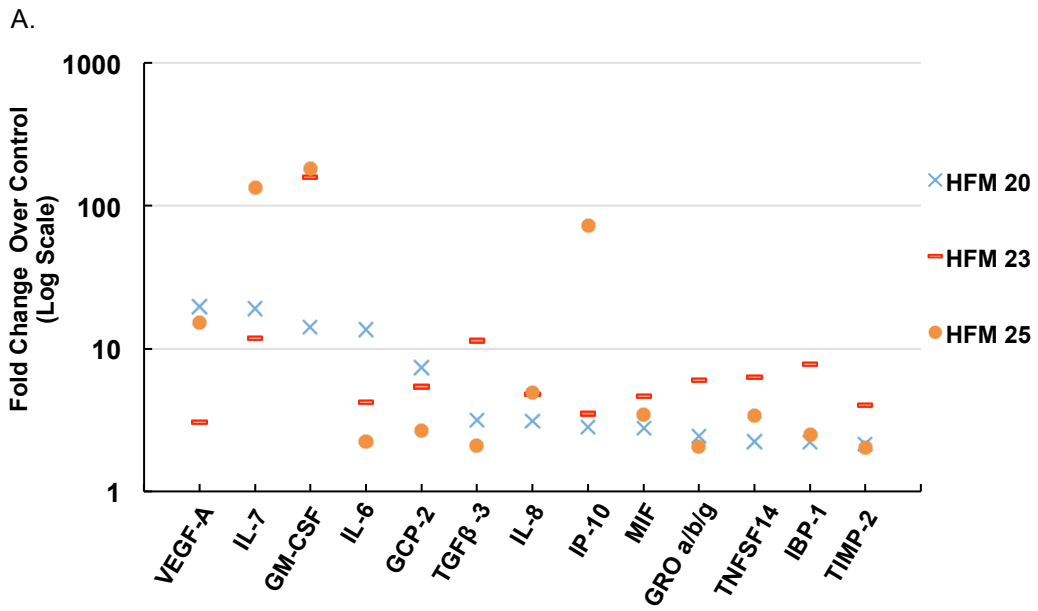

Figure 20. Altered Cytokine Secretion Profile post HIV-1 Infection. Cytokine secretions of microglia were examined using antibody array membranes. Fold changes of densitometry values from infected microglia normalized by untreated are graphed. Thirteen cytokines consistently elevated for three cases are shown. Abbreviations: Granulocyte chemotactic protein (GCP)-2; Macrophage migration inhibitory factor (MIF); CXC chemokines growth-regulated oncogene (GRO) a/b/g; TNF Super Family (SF) (TNF SF) 14; Insulin-like growth factor-binding protein (IBP) -1; Tissue inhibitor of metalloproteinase (TIMP)-2.

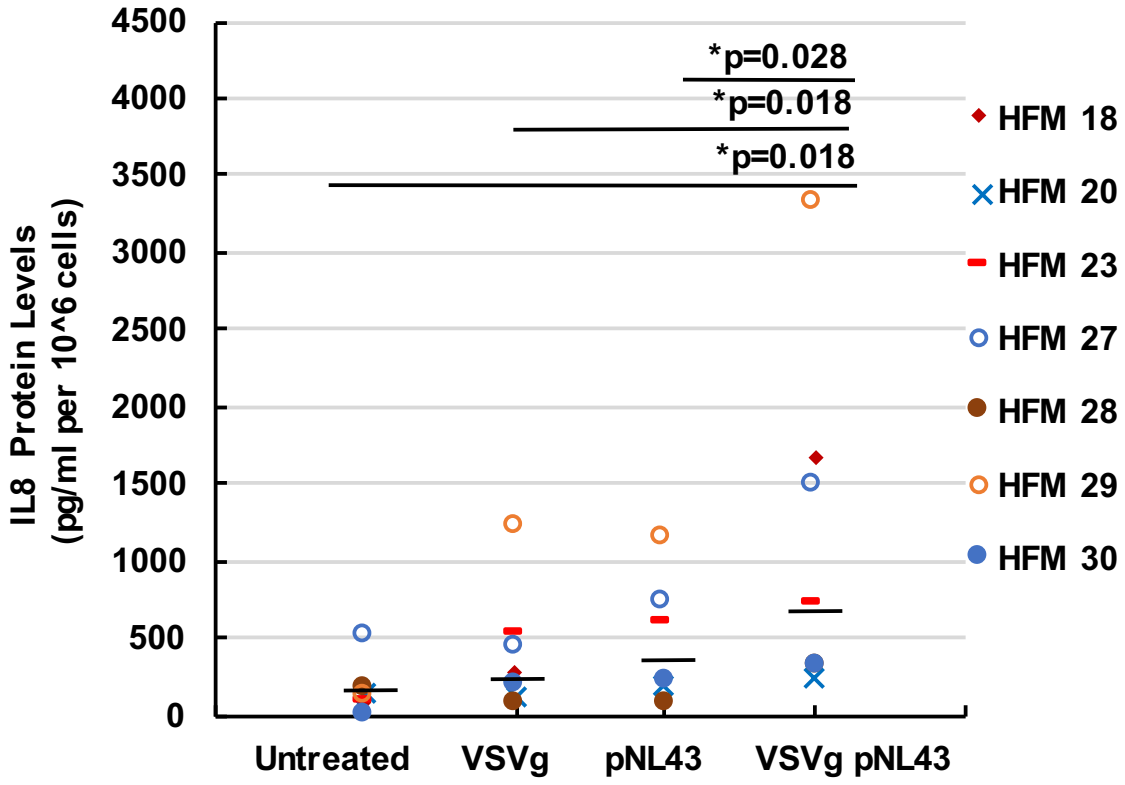

Figure 21. Elevated IL8 Secretion Post Infection. Secreted IL8 proteins in the supernatant were measured with ELISA for 7 cases. Medians for IL8 protein concentrations are: Untreated = 108; VSVg = 265; pNL43 = 411; VSVg pNL43 = 725. Nonparametric statistic analysis was performed and p values are annotated on the graphs. * indicates statistical significance.

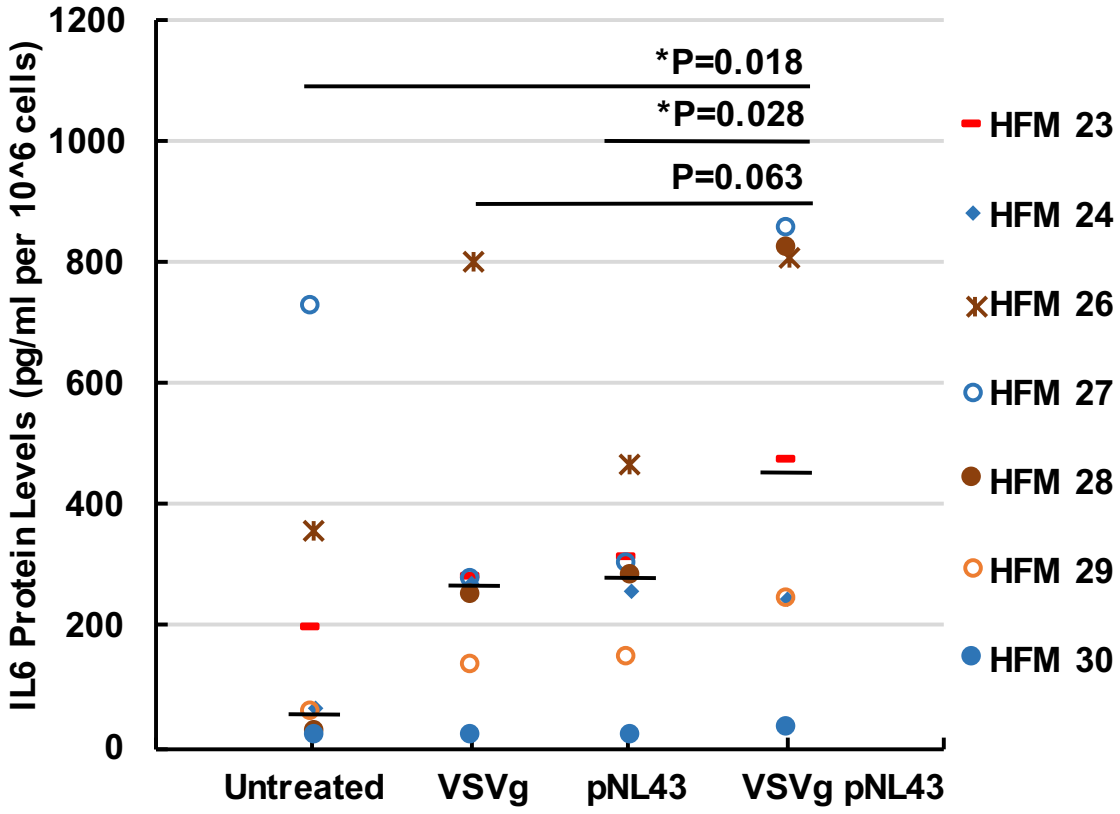

Figure 22. Elevated IL6 Secretion Post Infection. Secreted IL6 proteins in the supernatant were measured with ELISA for 7 cases. Medians for IL6 protein concentrations are: Untreated = 64.04; VSVg = 271.63; pNL43 = 284.01; VSVg pNL43 = 470.87. Nonparametric statistic analysis was performed and p values are annotated on the graphs. * indicates statistical significance.

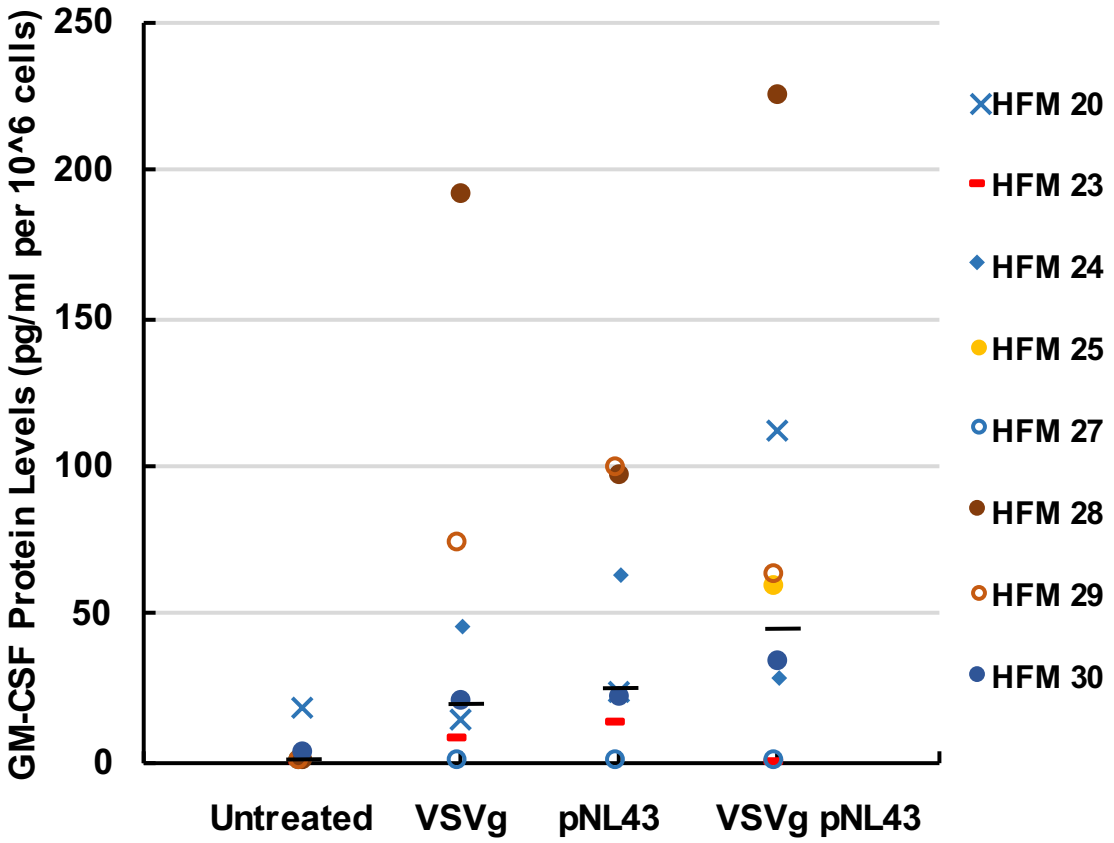

Figure 23. GM-CSF Secretion Post Infection. Secreted GM-CSF proteins in the supernatant were measured with ELISA for 8 cases. Medians for GM-CSF protein concentrations are: Untreated = 0; VSVg = 21.0; pNL43 = 23.9; VSVg pNL43 = 46.5. Nonparametric statistic analysis was performed.

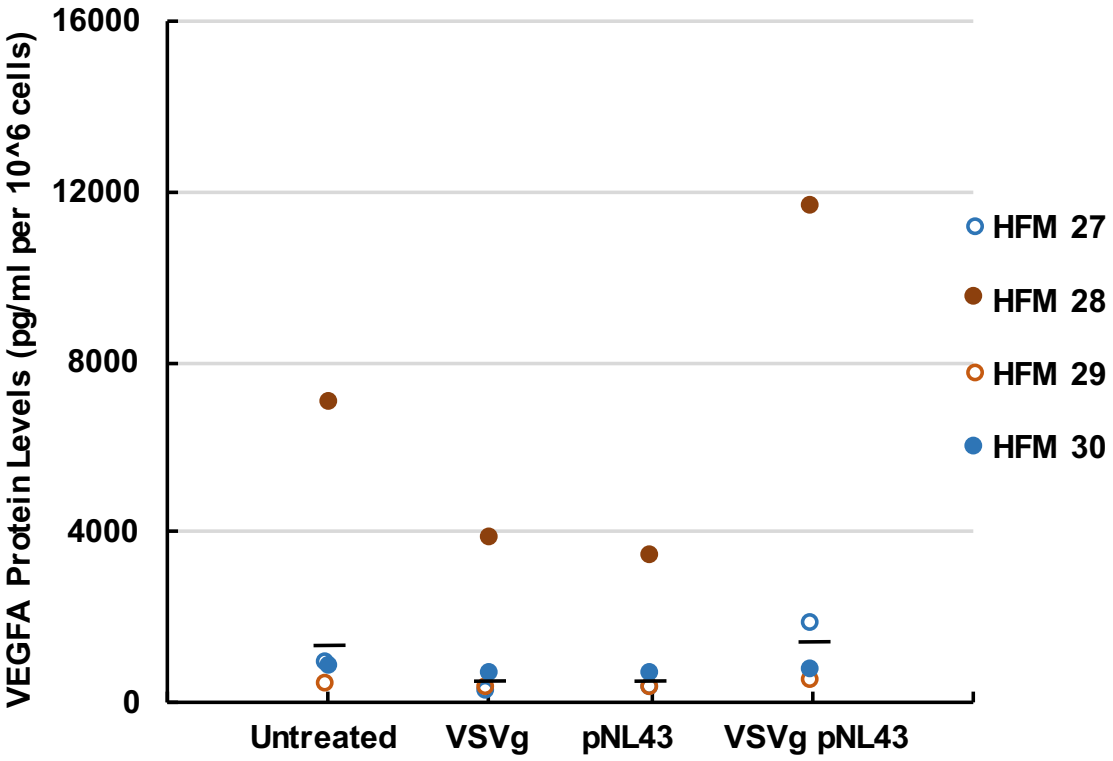

Figure 24. VEGF-A Secretion Post Infection. Secreted VEGF-A proteins in the supernatant were measured with ELISA for 8 cases. Medians for VEGF-A protein concentrations are: Untreated = 887; VSVg = 535.9; pNL43 = 546; VSVg pNL43 = 1319. Nonparametric statistic analysis was performed.

2.3.5 Nucleoside Treatments Attenuate Age-like Markers

Nucleosides (dNTP) treatments have been previously shown to prevent and reverse oncogene induced cellular senescence by restoring genomic stability (Aird et al. 2013). Since DNA damage response is elicited during HIV-1 infection of microglia, we proposed that dNTP treatment could ameliorate aspects of microglia age-like phenotype during HIV-1 infection. To test whether dNTPs could effectively prevent microglial age-like phenotype during infection, HFM were simultaneously incubated with dNTPs and VSVg pseudotyped HIV-1_{pNL43} viral particles. dNTP treatments reversed elevated p21 expression post HIV infection (Figure 25). Furthermore, dNTP treatments reduced IL8 secretion associated with HIV-1 infection at 250nM (Figure 26). We further confirmed that dNTP treatments did not affect HFM susceptibility to HIV-1 infection thus eliminating impaired infection as the mediating mechanism (Figure 27). These results suggest that dNTP could partially reverse selected aspects of HIV-1 infection induced age-like phenotype in microglia and could attenuate bystander effects of factors secreted by aging microglia.

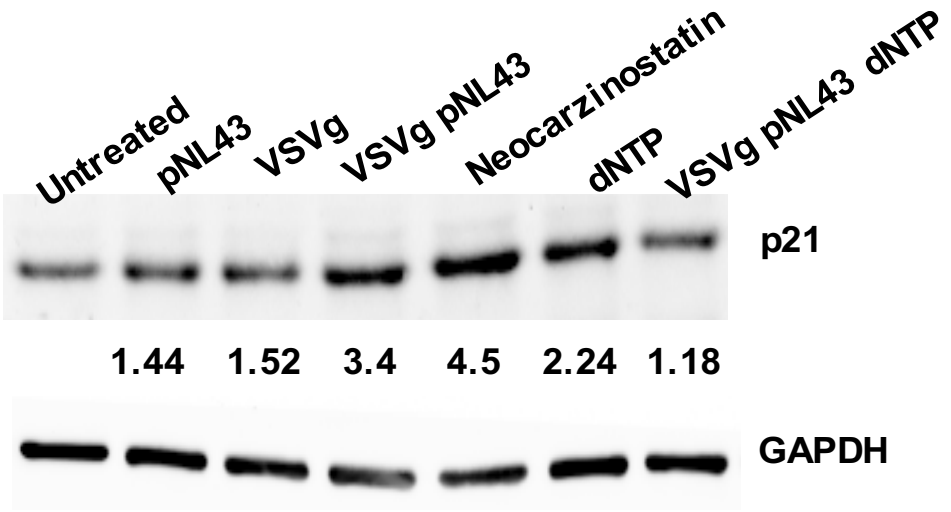

Figure 25. dNTP Treatment Reverses Elevated p21 Protein Levels. Immunoblot demonstrates elevated p21 levels in cells infected with VSVg pNL43 compared to controls (Untreated, pNL43, VSVg, and dNTP). Infected cells treated with dNTP (VSVG pNL43 dNTP) demonstrated attenuated levels of p21 compared to infected cells. Densitometries of p21 are normalized with endogenous control (GAPDH). Fold changes of p21 and for various treatment groups over untreated cells are annotated below the graphs.

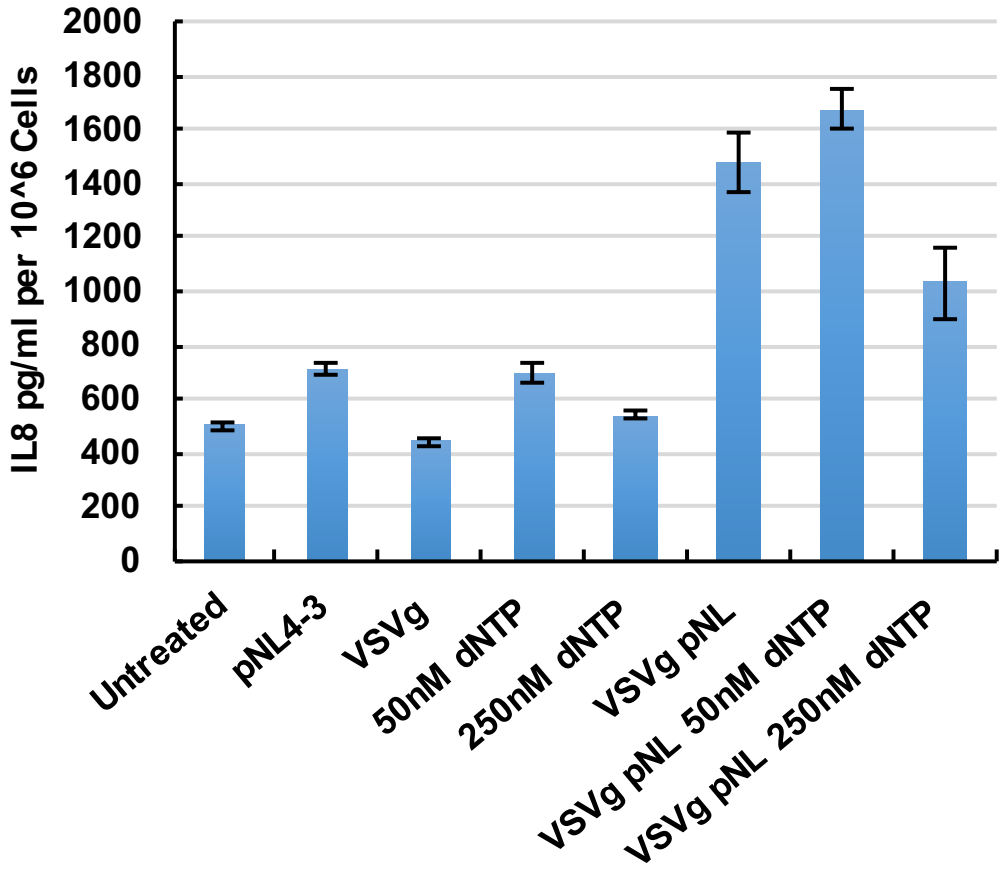

Figure 26. dNTP Treatment Reverse Elevated IL8 Secretion. ELISA demonstrates elevated IL8 secretion in cells infected with VSVg pNL43 compared to controls (Untreated, pNL43, VSVg, and dNTP). Infected cells treated with dNTP (VSVG pNL43 dNTP) demonstrate slightly attenuated IL8 release compared to infected cells. Concentration of IL8 protein is show in pg/ml and normalized per 10^6 cells. Result was obtained with HFM20. Since only one donor was used, no statistic analysis was performed.

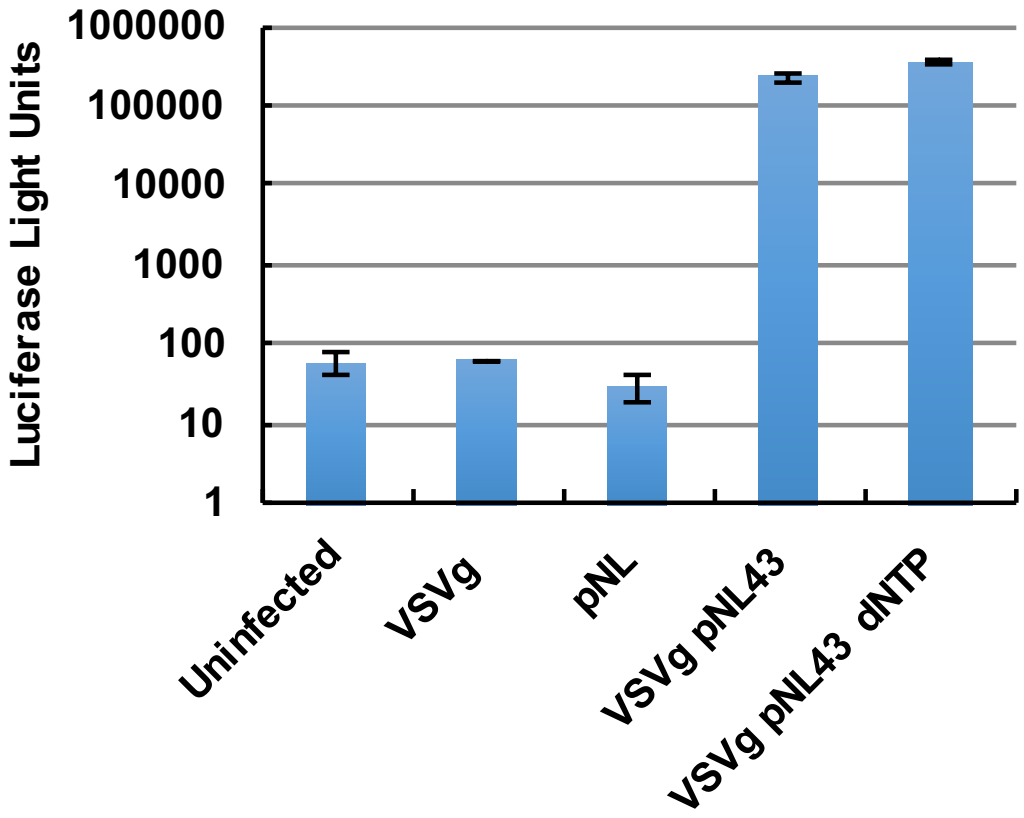

Figure 27. HFM Susceptibility to HIV-1 Infection. Control cells, infected cells and infected cells treated with dNTP were lysed and assayed for luciferase activities. Luciferase light units were graphed in log scale. dNTP treatment does not significantly impact HFM infectivity.

2.3.6 SASP induced Age-like Markers

Molecules secreted by senescent cells could modulate cellular functions of both senescent as well as non-senescent cells in the vicinity. It is known that components of SASP could both reinforce cellular senescence in cells that are already senescent as well as initiate cellular senescence programs in non-senescent cells (Meng et al. 2015). As a result, SASP could amplify the effects of senescent cells and contribute to systemic effects that are sometimes disproportionally widespread for the low number of senescent cells detectable in tissues. To examine whether components of SASP could illicit age-like responses in uninfected bystander microglia, we examined markers of microglial age-like phenotype including p21 and Caveolin-1 in microglia following incubation with supernatant from infected and control microglia. In addition to p21, which we have shown in Figure 1 to be up-regulated during HIV-1 infection, caveolin-1 protein expression was also examined because caveolin-1 has been shown to be elevated in various diseases associated with organismal aging and to regulate cellular senescence through activation of p53 pathway(Zou et al. 2011). Protein levels of both p21 and caveolin-1 were up-regulated in microglia exposed to supernatants from HIV-1 infected microglia compared to supernatants from control cells (Figure 28). Elevated levels of p21 and Caveolin-1 in supernatant transfer assay suggest that molecules secreted by aging microglia post HIV-1 infection could initiate the onset of age-like phenotypes in neighboring cells. Interestingly, the elevations in p21 and caveolin expression were attenuated in cells exposed to supernatant from infected cells that were also treated with nucleosides (Figure 28).

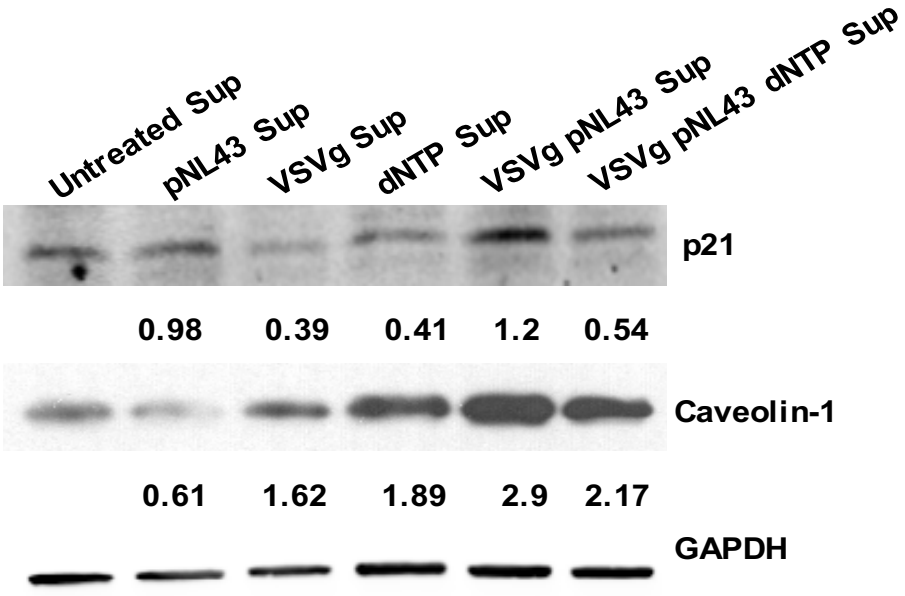

Figure 28. Supernatant Treatment Results in Elevated Age-like Markers. Immunoblot demonstrates elevated p21 and Caveolin-1 levels in cells incubated with supernatant from infected cells (VSVg pNL43 Sup) compared to controls (Untreated Sup, pNL43 Sup, VSVg Sup, dNTP Sup). Supernatant from infected cells treated with dNTP (VSVG pNL43 dNTP Sup) results in attenuated levels of p21 and caveolin-1 compared to infected cells. Densitometry of p21 and caveolin-1 was normalized with endogenous control (GAPDH). Fold changes of p21 and caveolin-1 for various treatment groups over cells incubated with supernatant from untreated microglia are annotated below the graphs.

2.3.7 Viability of HFM Post Treatment

Treatments do not significantly affect the viability of HFM. VSVg pNL43 infected HFM demonstrate 77% percent viable cells compared to around 88% viability of VSVg treated cells and 89% viability of untreated cells (Figure 29). Routine cell culture procedures such as trypsinization could have resulted in some cell death, which explains why HFM were not 100% viable. Cells treated with HIV-1 (77%) had around 10% lower viability compared to the controls (untreated: 89%; VSVg: 88%; pNL43: 84%), which suggests that the infection process does results in a low level of cell death. Neocarzinostatin treated cells displayed comparable viability (90%) to untreated cells showing that the concentration used does not cause cell death. Heat killed sample (half of the cells were killed at 100 °C for 10 minutes) only contained 19% viable cells, which demonstrates the validity of the assay.

A

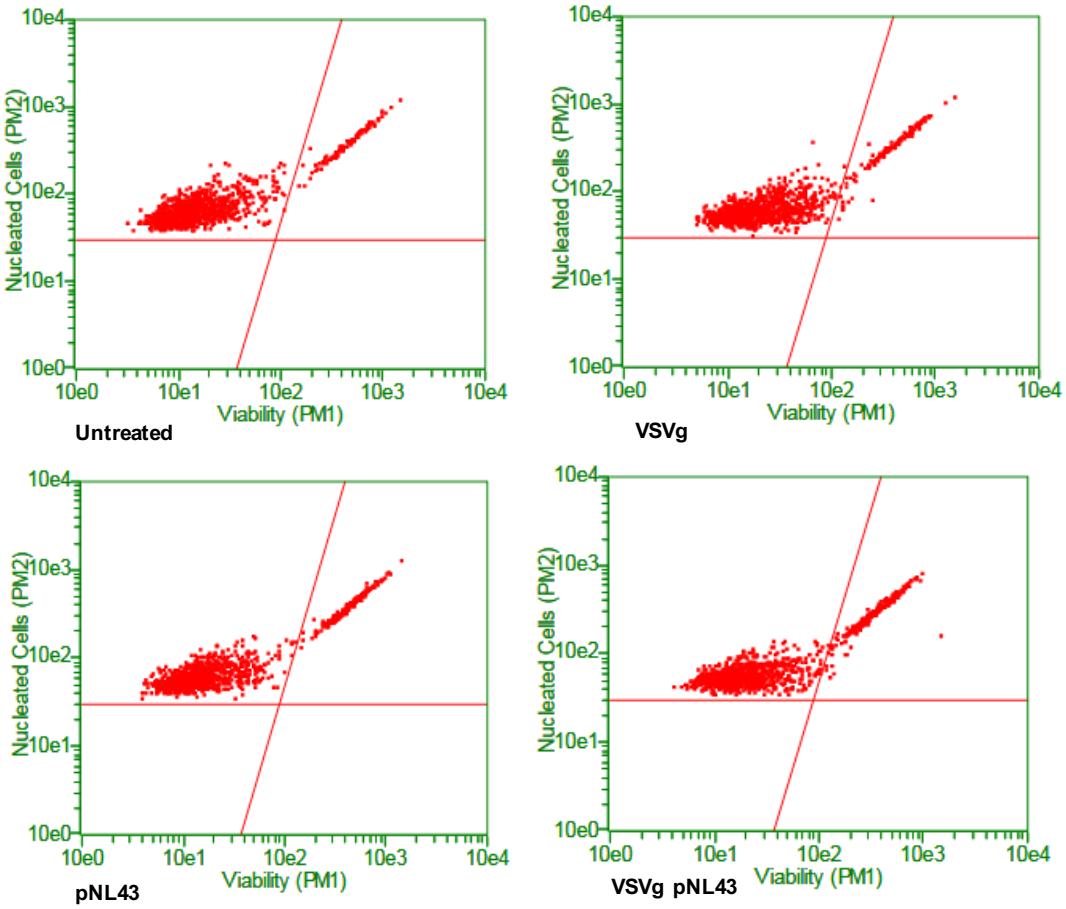

Figure 29. Viability Staining. Treated cells were assayed for viability with Guava ViaCount. Nucleated and viable cells appear in the upper left quadrant. Heat-killed sample was used as positive control. A&B. Representative graphs of viability staining. C. Percentages of viable cells were graphed.

B

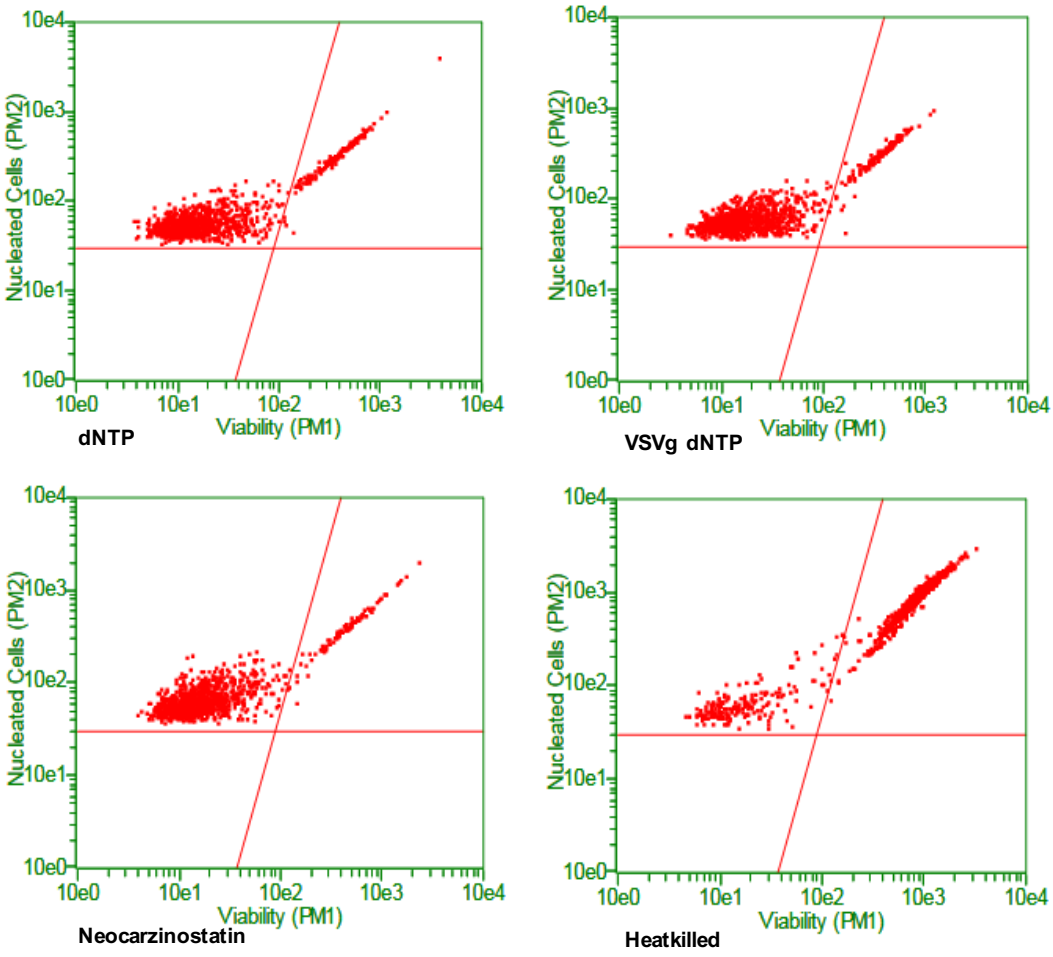

Figure 29. Viability Staining Continued. Treated cells were assayed for viability with Guava ViaCount. Nucleated and viable cells appear in the upper left quadrant. Heat-killed sample was used as positive control. A&B. Representative graphs of viability staining. C. Percentages of viable cells were graphed.

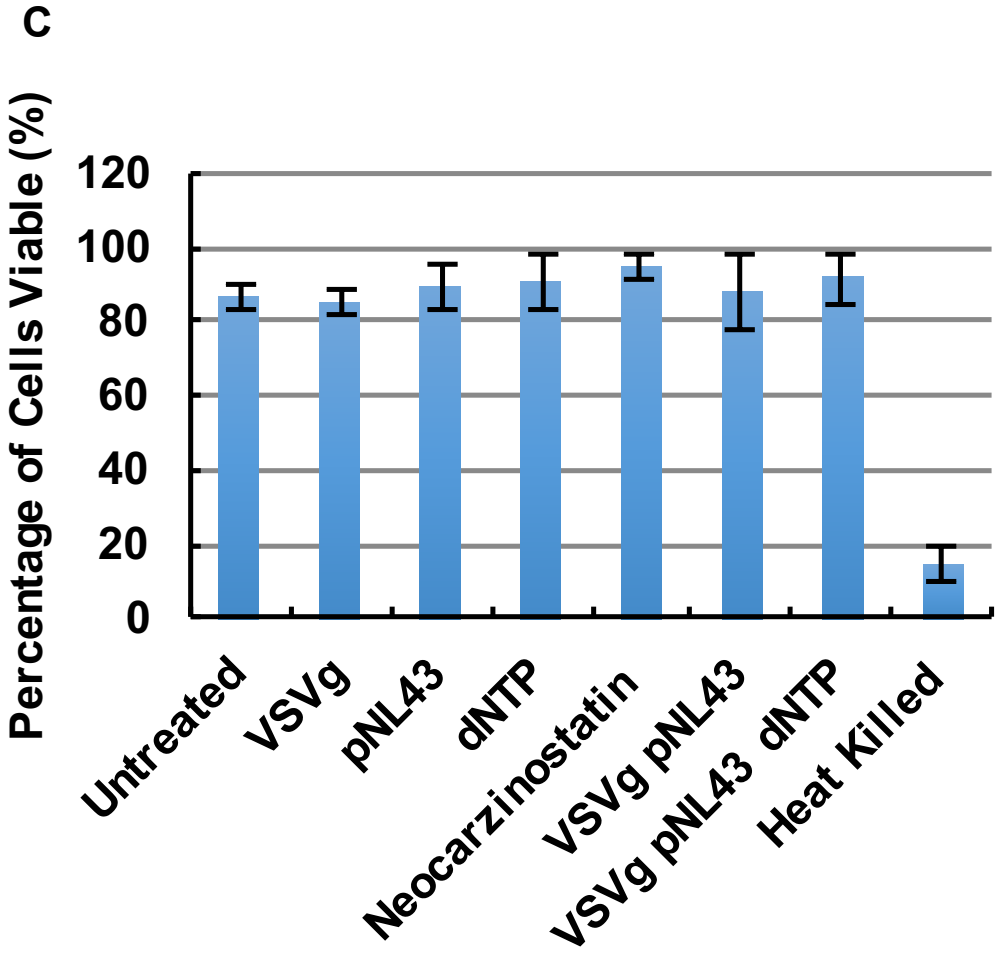

Figure 29. Viability Staining Continued. Treated cells were assayed for viability with Guava ViaCount. Nucleated and viable cells appear in the upper left quadrant. Heat-killed sample was used as positive control. A & B. Representative graphs of viability staining. C. Percentages of viable cells were graphed.

Chapter 3: Discussion

Various studies indicate that HIV-1 positive patients display advanced aging compared to their uninfected counterparts as demonstrated by changes in age-associated parameters including altered DNA methylation (Gross et al. 2016; Rickabaugh et al. 2015), reduced telomere stability (Chou et al. 2013; Liu et al. 2015) and elevated immune activation (Martin et al. 2013). Cellular senescence could underlie this accelerated biological aging. Within a HIV-1 infected cohort that has been previously shown to demonstrate advanced aging-associated frailty, premature cellular senescence characterized by lower endothelial cell density, was observed in HIV-1 positive patients compared to uninfected controls. The lower cell density was correlated with higher mRNA levels of cell cycle inhibitor CDKN2A known to mediate cellular senescence(Pathai et al. 2013b). Similarly, our results suggest that microglia age-like phenotype during HIV-1 infection could be an additional aspect of accentuated and/or accelerated aging of HIV-1 positive patients that could potentially contribute to the development of HAND during chronic HIV-1 infection (Figure 30).

In microglia cells, markers of organismal aging including percentages of cells positive for SA β-gal and protein level of p21 are elevated during HIV-1 infection suggesting the onset of microglial age-like phenotype (Figure 30). The elevated p21 protein levels in microglia post HIV-1 infection *in vitro* is consistent with the increased frequency of p21 positive glia cells detected in brain tissues of diseased patients with HAD(Jayadev et al. 2007). Elevated p21 during HIV-1 infection has also been previously observed in purified primary human CD4+ T cells. It was also shown in the same study that elevated p53 upstream of p21 promotes the induction of protein kinase R (PKR) that phosphorylates Tat. Phosphorylation of Tat suppresses Tat's function as HIV-1 transactivator, which ultimately results in the suppression of HIV-1 viral replication(Yoon

et al. 2015). In the present study, elevated level of p21 is observed post infection while elevated p53 protein expression is not detected. Since p21 expression is known to be regulated by the transcription factor p53, we speculate that p53 is also elevated but undetected in our system because it is only transiently increased at an earlier time point and is quickly degraded due to its naturally unstable structure (Tsvetkov et al. 2010). Given the significant role of p53 in regulating Tat activities, and subsequently HIV-1 viral replication, it would be of interest to examine mRNA and protein levels of p53 in future studies. It is possible that activation of cellular senescence during HIV-1 infection creates a cellular milieu unfavorable for active viral replication through, at least partially, inactivation of Tat in microglia cells. This would be of great interest especially considering microglia's potential role as cellular reservoir for HIV-1 in the post cART era. If true, cellular senescence could be a potential target for suppressing and ultimately eradicating HIV-1 from infected host cells. Finally, whether p21 regulates microglial function remains to be clarified. We could explore how altered p21 protein expression modulates microglial function by performing knockdown and/or overexpression experiments in untreated and infected cultures.

The age-like phenotype post HIV-1 infection is further bolstered by the observation of 53BP1 foci formation indicating activation of DNA damage response (Figure 30), which could then signal the cell cycle to be arrested by checkpoint proteins such as p21 in order to repair aberrant DNA replication. This is the hallmark of cellular senescence. Other markers of DNA damage response pathway including ataxia telangiectasia mutated (ATM), BRCA1, PCNA and γH2AX should be examined in conjunction with 53BP1 to more comprehensively analyze the activation, progression and completion of DNA damage repair. Elements of DNA damage response have not been extensively characterized in HAND tissues and could be done in future studies.

Mitochondrial dysfunction is closely associated with the development of cellular senescence(Salama et al. 2014). Altered mitochondrial mediated energy metabolism including reduced ATP synthesis could contribute to the progression of cellular senescence (Ziegler et al. 2015). Conversely, cellular senescence could result in mitochondrial dysfunction(Passos et al. 2006). We observed elevated mitochondrial ROS production as well as reduced mitochondrial respiration linked to ATP production in microglia during HIV-1 infection, both of which are aspects of mitochondrial dysfunction known to contribute to the development of cellular senescence (Figure 30). This change in mitochondrial function is also consistent with results of clinical studies with HIV-1 positive patients. Compromised mitochondrial ETC function has been observed in both treated and treatment-naïve HIV-1-infected patients (Miro et al. 2004; Miro et al. 2003). Moreover, abnormal mitochondrial morphology and mitochondrial dynamics have been observed in the brains of patients with HAD compared to HIV-1-infected but neurocognitively normal subjects (Fields et al. 2016b). We propose that mitochondrial dysfunction in aging microglia during HIV-1 infection may be a component of HAND development.

SASP development is a central aspect of cellular senescence. The most classic cytokines of SASP include IL6, IL8 and IL1. In particular, IL1 α has been shown to be an indispensable component of SASP regulating IL6/IL8 secretion during cellular senescence(McCarthy et al. 2013; Orjalo et al. 2009). As a result, it was perplexing that IL1 α was not detected in the supernatant post microglia infection. Recent evidence demonstrates, however, that mitochondrial dysfunction induces IL-1 independent-SASP profile that is significantly different than SASP induced by DNA damage(Wiley et al. 2016). Since mitochondrial dysfunction is observed in microglia during HIV-1 infection, although unclear whether it is a cause or effect, we stipulate that microglia age-like

phenotype during HIV-1 infection could be IL-1 independent considering the potential causal role of mitochondrial dysfunction in microglia aging during HIV-1 infection. The exact of role of mitochondrial dysfunction in the development of microglial age-like phenotype during HIV-1 infection needs to be clarified in future studies. Furthermore, it would of interest to distinguish between the effects of DNA damage and mitochondrial dysfunction, both of which are observed in microglia during HIV-1 infection, with regard to mechanistic pathways underlying SASP development.

In addition to IL6 and IL8, we also measured GM-CSF and VEGF-A levels with ELISAs since GM-CSF and VEGF-A were shown to be up-regulated on the antibody membranes. No statistically significant difference was detected between infected cultures and cultures exposed to VSVg and pNL43 control supernatants for both GM-CSF and VEGF-A indicating that the inductions of those two cytokines were not specific to infection and not part of the age-like phenotype. In addition to IL8, IL6, GM-CSF and VEGF-A, nine other secreted proteins were up-regulated in the infected cultures compared to untreated cells including IL7, IP10, GCP-2, MIF, (GRO) a/b/g, TNF SF14, IBP -1, TIMP-2 and TGF-3β, all of which play important roles in the regulation of immune and/or neuronal functions. There were also other cytokines that were altered in only two of the three donors examined and therefore not included in the list. We will selectively examine those cytokines with individual ELISAs as well with more donors. For instance, GRO a, a member of the GRO a/b/g family, is significantly up-regulated (71 and 155 fold increase over untreated) in two donor but not changed in the third donor. Considering the high magnitude of changes observed in two donors and its pertinence to aging (Fimmel et al. 2007) as well as neuronal health, we are very interested in confirming whether GRO a isoform is elevated in microglia post infection(Gordon et al. 2012).

Incubation with supernatant from infected microglia results in elevated p21 and caveolin-1 levels in treatment-naïve microglia (Figure 30). This observation elevates the physiological significance of current study since the percentage of infected microglia during natural infection could be very low in patients, while the consequence of such limited infection could be broad and lasting due to bystander effects as the results suggest. Additionally, elevated caveolin-1 levels in cells exposed to SASP could have significant ramifications with regards to HIV-1 infection and replication since it is known that HIV-1 infection affects caveolin-1 processing and conversely caveolin-1 restricts HIV-1 infection(Lin et al. 2015; Lin et al. 2012; van den Berg et al. 2014). Finally, caveolin-1 has been shown to modulate various aspects of microglial function including amyloid beta phagocytosis, microglia activation and microglial mitochondrial respiration(Jang et al. 2015; Niesman et al. 2013). As a result, altered caveolin-1 expression could have significant effects on various aspects of microglial function contributing to HAND development.

It is also interesting that nucleosides treatment reverses elevated p21 levels post infection as well as p21 and caveolin-1 levels post exposure to supernatant of infected cells, suggesting that dNTPs suppresses senescence inducing factor(s) in SASP. As a proof of concept, IL8 levels were reversed in the dNTPs treated cells post infection. Since IL8 may not be the factor that is responsible for the induction of p21 and caveolin-1 in the supernatant transfer assay, future studies are required to determine the specific mechanisms underlying SASP-induced elevation in p21 and caveolin-1. In addition, further experiments are required to elucidate the mechanisms of action of the nucleosides. Nucleosides are used instead of corresponding nucleotides because nucleosides can be taken up across the cell membrane through nucleoside transporter proteins(King et al. 2006). Considering the dramatic impairment of ATP-linked

mitochondrial respiration in microglia during HIV-1 infection, it is possible that impaired ATP production contributes to the development of microglia senescence. This should be confirmed by measuring the content of dNTPs post infection in future experiments. As a result, we propose that the nucleoside adenosine, which is an ATP precursor, might be the principle mediator in preventing senescence-like phenotypes. Future studies should examine whether adenosine independently prevents the onset of age-like phenotype associated with HIV-1 infection in microglia cells. Furthermore, since adenosine is an important neurotransmitter regulating various microglial functions including phagocytosis and migration through activation of p1 purinergic receptors expressed on the surface of human microglia cells (Koizumi et al. 2013), it is important to clarify whether adenosine modulates microglial phenotypes through direct activation of purinergic receptors instead of contributing to the synthesis of intracellular ATPs.

To mechanistically assess whether HIV-1 infection indeed induces the age-like phenotypes observed, anti-retroviral treatments targeting various steps of the viral life cycle will be utilized to prevent the onset of age-like phenotypes. This intervention will help elucidate whether specific steps in viral infection and/or replication facilitate the aging process. A potential complication of this intervention is that various studies demonstrated anti-retroviral treatments could contribute to the accelerated/accentuated aging phenotype in HIV-1 infected population (Payne et al. 2011). As a result, it is important that we titrate the compounds to concentrations that would prevent viral infection without inducing age-like phenotypes.

Another potential mechanism that we are interested in examining is the regulation of microglial function by microRNAs (miRNAs). miRNAs are small non-coding RNAs that regulate gene expression on the post-translational level. miRNAs have been shown to regulate immune activation and cellular senescence, both of which are relevant

processes during HIV-1 infection. Selected miRNAs such as miR124 and miR155 have been shown to regulate immune activations including those of microglia cells, while others have been shown to regulate the onset of cellular senescence including let7 and miR146a/b (Benhamed et al. 2012; Bhaumik et al. 2009; Ponomarev 2011; Tarassishin et al. 2011). miRNA profiles could be altered during HIV-1 infection, which could then subsequently regulate functional changes in microglia including activation and senescence. Altered miRNA profile could be a mechanism mediating the onset of age-like phenotype in microglia during HIV-1 infection. As a result, we plan to assess how HIV-1 infection could affect microglial miRNAs during HIV-1 infection. Many studies have detected changes in miRNA profiles in HIV infected patients. Houzet et al demonstrated that five highly T cell specific miRNAs are significantly down-regulated including miR150, miR191, miR223, miR16 and miR146b in HIV-1 infected patients (Houzet et al. 2008). Furthermore, various groups demonstrate that specific miRNA signatures might differentiate patients with different patterns of disease progression. It was shown that members of miR29, miR125b, 150, and 31 are differentially expressed in HIV elite suppressors from viremic patients (Witwer et al. 2012). Swaminathan S et al show that let7b and let7f are significantly reduced in infected patients compared to elite suppressors (Swaminathan et al. 2012). Study of miRNA profiles of elite suppressors, multiple expose uninfected and naïve patients showed that miR155 is the only miRNA that could differentiate elite suppressors from native controls(Bignami et al. 2012). Disturbance of miRNAs involved in the apoptotic pathway is detected in a group of rapid progressors compared to chronic progressors according to a study by Zhang et al (Zhang et al. 2013). In addition to the association with overall disease progression during HIV-1 infection, specific miRNA signatures is also shown to correlate with the development of HAND through analysis of brain tissues, CSF or plasma. Witwar et al examined the plasma miRNAs of SIV infected pigtail macaques and demonstrated

significant changes in 45 miRNAs. Five of which, including miR146a, correlated with severe CNS diseases and additionally, let7e level correlated with IL6 level which is associated CNS inflammation(Witwer et al. 2011). Furthermore, 11 miRNAs are significantly up regulated in the CSF of patients with HIVE compared to that observed in patients without encephalitis (Pacifici et al. 2013). miRNA regulation of CNS integrity in the context of HIV-1 infection are cell type specific. In brain sections of SIV infected rhesus monkeys, FISH and IF results indicate that miR-142 is up regulated in MAP2+ neurons. Elevated miR-142 leads to reduction in SIRT1 expression and consequently reduced expression of monoamine oxidase, which could negatively affect neurotransmitter production(Chaudhuri et al. 2013). HIV infection could result in elevated miR21 in neurons, which could affect potassium current through inhibiting MEF2(Yelamanchili et al. 2010). Analysis of frontal lobes of HIVE brain tissues indicates down-regulation of miR129-3p and miR130a astrocytes, which contribute to increased level of apoptosis mediated by caspase 6 activation (Noorbakhsh et al. 2010). Details are presented in Table 1. In vitro studies of cell culture models post HIV-1 infection detect changes in miRNA profiles in HIV-1 infected cells compared to controls using different cell types including PBMCs, astrocyte, microglia and neurons. Deep sequencing of PBMCs infected with BAL at 27 hours post infection detected significant change (>2 fold increase) in only one miRNA-– miR30b-5p(Whisnant et al. 2013). This in vitro study contradicts previous reports that detect changes in multiple other miRNAs (Chang et al. 2013; Triboulet et al. 2007). This discrepancy could be due to several factors: different cells types, (PBMC vs. Jurkat) and different time point post infection. Overall, the data suggest that HIV infection could modulate host miRNAs on the cellular level. Different miRNA profile is detected in vitro compared to what was observed in clinical samples, which suggests that environmental factors other than infection including cytokine secretion could modulate host miRNAs. Various studies indicate that distinct

miRNAs correlate with disease progression and specifically severity of CNS disorders. miRNAs have the potential to be used as biomarkers for HIV/AIDS pathogenesis and HAND development. In addition, miRNAs could serve as therapeutic targets. In particular, antagomir that targets miR122 has shown clinical promise for the treatment of hepatitis C infection(Janssen et al. 2013). Targeting miRNA is a viable approach for cancer treatment based on preclinical data(Velu et al. 2013). Additionally, animal models are being used to explore whether microRNA antagomirs can be used to treat neurological disorders in murine models of ischemic stroke and ALS. Although delivery of compounds targeting miRNAs through introcerebroventricular injections is a viable option for animal models as described in those publications, alternative approach should be fully explored in order to limit treatment complications in patients in a clinical setting (Koval et al. 2013; Selvamani et al. 2012). In addition, post treatment analysis of the murine models indicates that antagomirs are detectable in multiple cell types of the CNS including neurons, astrocytes and microglia cells. miRNA therapy could be explored as a potential safe and effective therapeutic approach for treating HAND in the context of aging of the population.

Since we are working with primary human cells, significant donor-dependent variations occur in various phenotypes observed. Some donors would exhibit very strong age-like qualities consistently across phenotypes such as HFM 20, which exhibits elevated percentages of cells positive for SA- β-gal, elevated p21 levels, elevated IL8, reduced basal OCR and reduced ATP linked respiration. dNTP was shown to prevent p21 and IL8 elevation post infection in HFM 20. Other donors, such as HFM 26, only exhibit one or two age-like phenotypes post infection. HFM 26 demonstrates increased SA- β-gal positivity, but no change in p21 or IL6 levels post infection. It is possible that the senescence program is activated to different extend in different donors. It is also

important to note that not all phenotypes were examined for every single donor. Depending on the number of cells available, selected phenotypes were examined for a particular donor.

The biggest caveat of the present study is the low statistical significance for some experiments due to high variability in results obtained with different cases of primary human microglia, which could result from three pitfalls in experimental design and execution: 1. variability of microglia from different brain regions; 2. variable infectivity of different batches of pseudotype HIV-1; 3. technical inconsistencies. Firstly, recent studies demonstrate region-dependent gene expressions of murine microglia, which supports the speculation that microglia derived from different brain regions have variable functions(Grabert et al. 2016). If different cases of microglia were obtained from different brain regions, potential region dependent functional differences could have contributed to the variable results we see. Unfortunately, thus far I have not read a paper that utilized human microglia from specific regions of the brain for functional studies (including gene expression on the protein level) due to the limited amount of cells obtained from each tissue preparation. Hopefully, with advancement in primary cell extraction from human tissues, we will be able to examine microglia from specific brain regions and thus reducing the variability between independent experiments. Secondly, pseudotype HIV-1 virus from different preparations can have very different infectivity indicated by different luciferase levels, which can contribute to varying percentages of cells being infected in a primary microglia culture. Since we hypothesize that HIV-1 infection is the stressor inducing the aging phenotype in microglia, inconsistent infection could lead to variable results. To remediate this source of experimental variability, we could scale up to produce enough virus for all experiments in one setting. Lastly, although the variability in results could be donor dependent, we have not eliminated

technical inconsistencies by repeating experiments with one donor. Given the region dependent differences mentioned above, cells from same donor could still present intrinsic differences.

Although the use of single-round infectious virus provides a clean model to examine the effect of HIV-1 infection upon first exposure, the model does not re-capitulate native infection, in which infectious virions are produced and can infect other cells. In future experiments, we will perform follow-up studies utilizing fully infectious virus.

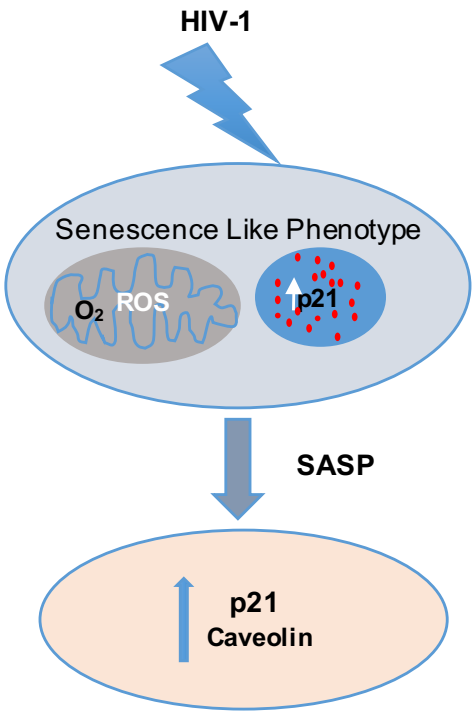

Figure 30. Working Model. Microglia develop various senescence-like phenotypes during HIV-1 infection including elevated SA- β-gal positivity, p21 protein expression and formation of 53BP1 DNA damage foci. Altered mitochondrial functions including elevated mitochondrial ROS production and impaired ETC respiration are associated with the development of senescence like phenotypes. Increased cytokine production characteristic of senescence associated secretory phenotype is also detected. Components of supernatant post infection leads to the development of senescence markers

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APPENDICES

Appendix A
Tables of p-Values

SA-β-gal

Paired Comparison	VSVg	pNL43	VSVg pNL43	Neocarzinostatin
Untreated	0.345	0.273	0.018	0.008
VSVg		1.000	0.080	
pNL43			0.068	

p21

Paired Comparison	VSVg pNL43
VSVg	0.305
pNL43	0.046
Neocarzinostatin	0.705

IL8

Paired Comparison	VSVg	pNL43	VSVg pNL43
Untreated	0.176	0.075	0.018
VSVg		0.249	0.018
pNL43			0.028

IL6

Paired Comparison	VSVg	pNL43	VSVg pNL43
Untreated	0.237	0.310	0.018
VSVg		0.499	0.063
pNL43			0.028

GM-CSF

Paired Comparison	VSVg	pNL43	VSVg pNL43
Untreated	0.046	0.028	0.043
VSVg		0.345	0.463
pNL43			0.753

VEGF-A

Paired Comparison	VSVg	pNL43	VSVg pNL43
Untreated	0.068	0.068	0.273
VSVg		0.715	0.068
pNL43			0.068

Mitochondrial ROS

Paired Comparison	VSVg	pNL43	VSVg pNL43
Untreated	1.000	0.273	0.005
VSVg		0.465	0.068
pNL43			0.273

Basal OCR

Paired Comparison	VSVg	pNL43	VSVg pNL43
Untreated	0.068	0.144	0.249
VSVg		0.893	0.043
pNL43			0.080

ATP -linked OCR

Paired Comparison	VSVg	pNL43	VSVg pNL43
Untreated	0.465	0.068	0.028
VSVg		0.043	0.043
pNL43			0.080

Maximal OCR

Paired Comparison	VSVg	pNL43	VSVg pNL43
Untreated	1.000	0.465	0.116
VSVg		0.225	0.043
pNL43			0.500

Pronton Leak

Paired Comparison	VSVg	pNL43	VSVg pNL43
Untreated	0.068	0.465	0.753
VSVg		0.893	0.225
pNL43			0.500

Spare OCR

Paired Comparison	VSVg	pNL43	VSVg pNL43
Untreated	0.068	0.068	0.173
VSVg		0.138	0.686
pNL43			0.225

Appendix B
List of Abbreviations

Alzheimer's disease (AD)
Amyloid precursor protein (APP)
Apolipoprotein E (ApoE)
Asymptomatic neurocognitive impairments (ANI)
Ataxia telangiectasia mutated (ATM)
Autophagy-related factors (Atg)
B-cell lymphoma (Bcl)-2
BCL2 Associated Athanogene (BAG) 3
BCL2-associated X protein (Bax)
Beta amyloid (Ab)
Cerebrospinal fluid (CSF)
Cu/Zn superoxide dismutase (SOD1)
CXC chemokines growth-regulated oncogene (GRO) a/b/g
Cyclooxygenase (Cox)-2
glutathione peroxidase (GPx1)
Granulocyte chemotactic protein (GCP)-2;
HIV Associated Neurocognitive Disorder (HAND)
HIV-1 encephalitis (HIVE)
HIV-1-associated dementia (HAD)
Human Immunodeficiency virus (HIV)
Human leukocyte antigen-D-related (HLA-DR)
IFNg-induced protein (IP)-10
Insulin-like growth factor-binding protein (IBP) -1
Intercellular adhesion molecule (ICAM)-1
Interferon (IFN) g
Interleukin (IL)-4
Ionized calcium-binding protein-1 (Iba-1)
Lipopolysaccharide [LPS]
Macrophage inflammatory protein (MIP)-1b
Macrophage migration inhibitory factor (MIF)
Magnetic resonance imaging (MRI)
Major histocompatibility complex II (MHC II)
microRNAs (miRNAs)
Murine double minute 2 (MDM2)
N-methyl-D-aspartate (NMDA)
Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases
Nitric oxide synthase (iNOS)
Nucleosides (dNTP)
p53 binding protein 1 (53BP1)
Positron emission tomography (PET)
Pre-integration complex (PIC)
Proliferating cell nuclear antigen (PCNA)
Reactive oxygen species (ROS)
Retinoblastoma susceptibility gene product (pRB)

Senescence Associated Secretory Phenotype (SASP)
Senescence Associated β-gal (SA- β-gal)
Severe combined immunodeficiency (SCID)
Simian immunodeficiency virus (SIV)
Tissue inhibitor of metalloproteinase (TIMP)-2.
TNF Super Family (SF) (TNF SF) 14;
Toll-like receptors (TLR)
Transforming growth factor b (TGF-b)
Tumor necrosis factor (TNF) a
Vascular Endothelial Growth Factor (VEGF)-A

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